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(54) Title: METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL MEMBRANE PROTEINS

(57) Abstract

Methods and agents are provided to decrease or increase the migration of dendritic cells for the suppression or enhancement, respectively, of the development of immunity and the immune response, by modulating the dendritic cell membrane proteins p–glycoprotein (MDR–1) and tissue factor. Agents which suppress migration have utility in the treatment of immunologically–mediated and inflammatory diseases such as graft rejection, contact dermatitis, seasonal allergies, asthma, and food allergies. Agents which enhance migration are useful for increasing the effectiveness of vaccines. Agents are also disclosed which enhance the migration of monocytes, useful in the treatment of chronic inflammatory diseases. Methods are also provided for identifying useful agents by measuring the effect on dendritic cell migration of agents which modulate p–glycoprotein and tissue factor activity, as well as the effect of agents on monocyte migration.

METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND

INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL

MEMBRANE PROTEINS

RESEARCH SUPPORT

FIELD OF THE INVENTION

10   The present invention relates generally to methods and agents for enhancing or suppressing

the development of immunity and the immune response by modulating the migration of

dendritic cells, and methods for identifying agents with migration modulating activity.

Methods and agents for the enhancement of monocyte migration out of chronic inflammatory

foci are also provided.

BACKGROUND OF THE INVENTION

15

The development of immunity to a specific antigen and the subsequent immune response on

re-encountering that antigen involve multi-step interactions between several immune cell

types and co-stimulatory molecules with critical spatial and temporal components. In brief,

an antigen encountered by antigen-presenting cells is taken up, processed, and its peptides

20   subsequently prime T lymphocytes to generate antigen-specific T lymphocytes (e.g.,

cytoxic and memory cells), and through the interaction with B cells, result in the formation

of antigen-specific antibodies. Immune responses are initiated after antigen-presenting cells,

particularly dendritic cells (DC), capture antigens in organs such as skin, lung and gut, and

migrate via afferent lymphatic vessels to draining lymph nodes. T lymphocytes continually

5   recirculate through lymph nodes, so the newly arrived, antigen-bearing DC become

positioned to select lymphocytes that bear receptors for the presented antigens (1). For

example, when skin is transplanted (2), or a contact allergen such as poison ivy is applied (3),

epidermal DC (also termed Langerhans cells) migrate from their epithelial locations and carry

antigens or allergens to the lymph node. If the lymphatic conduits to the draining lymph

10   nodes are severed, the immune system does not become primed to the antigen (2). As they

migrate, the DC undergo maturation, increasing their expression of molecules involved in

antigen presentation, including major histocompatibility complex (MHC) II products, CD80

(B7-1), and CD86 (B7-2) (4, 5).

Thus, dendritic cell migration is a critical step in both the induction of immunity and in the

15   immune response. Dendritic cell migration is essential for the successful induction of an

immune response to an administered vaccine, for eliciting antibody production and cell-

mediated immunity against infectious microorganisms and arising neoplastic cells within the

body. Likewise, dendritic cell migration is responsible for diseases involving an exaggerated,

inappropriate or undesired immune response such as allergic asthma, food allergies, contact

20   dermatitis, seasonal allergies, and autoimmune diseases such as psoriasis, in which known or

as-yet undescribed antigens are transported to T lymphocytes.

White blood cells known as monocytes are precursors of dendritic cells and tissue

macrophages. These cells migrate throughout the tissues, and may develop into dendritic

cells, as described above, or macrophages, which remain in the tissues. Excessive retention

and pro-inflammatory activity of macrophages in certain tissues is implicated in acute and

5   chronic inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and

granulomatous diseases.

Blockage of or interference with dendritic cell migration interferes with the immune

response. Blockage has been achieved by the use of antibodies against certain dendritic cell

proteins. Before leaving the epidermis, dendritic cells down-regulate E-cadherin, allowing

10   retraction from neighboring keratinocytes (31). In the presence of anti-α_6 integrin antibody,

epidermal DC up-regulate MHC II and retract from neighboring keratinocytes, and fail to

migrate out of the epidermis (32). Another adhesion molecule, CD44, is also used by DC

during mobilization from the epidermis (33).

It is towards improved means for specifically modulating the migration of dendritic cells

15   from the periphery to lymphatic vessels for both the positive and negative control of the

immune response, and blockage of monocyte migration to control the inflammatory response,

that the present invention is directed.

The citation of any reference herein should not be deemed as an admission that such

reference is available as prior art to the instant invention.

20   SUMMARY OF THE INVENTION

The inventors herein have made the surprising and unanticipated finding that certain dendritic

cell membrane proteins, namely p-glycoprotein, also known as MDR-1, the transporter

responsible for multi-drug resistance, and tissue factor (TF), a procoagulant protein, are

involved in the migration of dendritic cells (DCs) from the peripheral tissues to the lymphatic

5   vessels. Alteration of dendritic cell migration activity by manipulating these surface proteins

is useful for both enhancing and suppressing the immune response. Furthermore, the

migration of monocyte-derived cells out of tissues has been found to be inhibited by agents

which interfere with monocyte p-glycoprotein.

Thus, in accordance with the present invention, methods for the modulation of immunity and

10   the immune response in a mammal is provided by an agent which is capable of interacting

with the dendritic cell membrane proteins p-glycoprotein and tissue factor, and altering the

migration of dendritic cells from the peripheral tissues to the lymphatic vessels. In one

embodiment, modulation results in decreasing the migration of dendritic cells and hence the

suppression of immunity or of an immune response. Agents useful for the practice of this

15   aspect of the invention include antibodies and antibody fragments which bind to p-

glycoprotein, agents which block transport activity of p-glycoprotein, antisense

oligonucleotides to p-glycoprotein and antagonists of p-glycoprotein. Preferred agents are

those which reverse multi-drug resistance, such as calcium channel blockers, reserpine,

certain antimalarial drugs, cyclosporins, phenothiazines, tamoxifen and its metabolites,

20   certain antibiotics, antisense oligonucleotides, and other compounds known to antagonize the

activity of p-glycoprotein. Useful antibodies include MRK-16, UIC2, 4E3, and their

fragments.

In a further embodiment, antagonists of tissue factor are useful for the suppression of the development of immunity and of the immune response. Such antagonists include antibodies and antibody fragments to tissue factor, tissue factor antisense oligonucleotides, fragments of tissue factor, and compounds known to antagonize tissue factor such as dilazep and retinoic

5   acid.

Suppression of the immune response is useful for the treatment of several diseases and conditions. For example, allergic reactions, graft-vs-host disease, transplant rejection, autoimmune diseases, progression of HIV infection, contact dermatitis, and food allergy are treatable by the methods and agents of the present invention. The agents may be

10   administered by appropriate routes including topical, parenteral, ophthalmic, nasal, pulmonary, etc., for delivery to the site of encounter of the dendritic cells with the antigen and to prevent their subsequent migration to the lymphatic vasculature.

In a further embodiment, certain methods and agents of the present invention may be used which enhance the migration of dendritic cells from the peripheral tissues to the lymphatic

15   vessels. These methods and agents promote the development of immunity or of an immune response to a newly encountered antigen and enhance immunity or the immune response on re-encountering an antigen. Agents useful in the practice of this aspect of the present invention are agonists of the dendritic cell membrane proteins p-glycoprotein and tissue factor. Examples of useful agents include bromocriptine, N-acetyl-leucine-leucine-tyrosine

20   amide, and cytarabine for p-glycoprotein. In a preferred embodiment, the agent is co-administered with an immunogen to enhance the development of an immune response to said immunogen, for example, when the immunogen is a vaccine.

In a further aspect of the present invention, methods for the identification of agents useful for the modulation of dendritic cell migration is provided. These methods comprise the steps of first identifying an agent capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, followed by determining the extent of modulation of the

5   migration of dendritic cells in vitro or in vivo by the agent. These methods identify agents useful for modulating the development of immunity or an immune response.

In another aspect of the present invention, methods for the modulation of inflammation in a mammal are provided by an agent which is capable of enhancing the function of the monocyte membrane protein p-glycoprotein, and increasing the migration of inflammatory

10   monocytic cells from the tissues. Agents useful for the practice of this aspect of the invention include the aforementioned agonists of p-glycoprotein. Increasing monocyte migration out of tissues is useful in the treatment of such inflammatory conditions as atherosclerosis, rheumatoid arthritis and granulomatous diseases.

In a still further aspect of the present invention, methods for the identification of agents

15   useful for the augmentation of monocyte migration out of tissues is provided. These methods comprise the steps of first identifying an agent capable of enhancing the monocyte membrane protein p-glycoprotein, followed by determining the extent of augmentation of the migration of monocytes in vitro or in vivo by the agent. These methods identify agents useful for treating inflammatory conditions and diseases.

20   These and other aspects of the present invention will be better appreciated by reference to the following figures and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 A-B. Effect of anti-MDR-1 monoclonal antibodies (mAbs) on transendothelial migration of mononuclear phagocytes. **(A)** Monocytes were allowed to migrate across endothelial monolayers with or without addition of anti-MDR-1 mAb MRK16, or mAb to b_2

5 integrin (IB4) or CD31 (hec 7). Cultures were incubated for 1.5 hr. **(B)** To examine subsequent basal-to-apical transmigration, monocytes were first allowed to accumulate beneath the endothelium in the absence of added mAb for 1.5 hours, followed by addition of mAbs (all IgG2a; MRK16 used as $F(ab')_2$ fragments) at the indicated concentrations; in some samples, monocytes were preincubated with MRK16 or isotype-matched control mAb hec 1

10 against cadherin 5 (Pre-MRK16 and Pre-Hec 1) before addition to the endothelium. After 48 hr, cultures were fixed for analysis. Data are plotted relative to control levels of reverse transmigration (no mAb added), in which 50% of the subendothelial MP retraverse the endothelium in 48 hr. Bars represent means ± SD from three to ten experiments, except UIC2 which was tested in a single experiment.

15 **Figure 2.** Effect of anti-MDR-1 mAb on emigration of DC and T lymphocytes from skin explants. Explants of human skin were floated in culture medium without added mAb (n = 10) or in medium containing anti-MDR-1 mAbs MRK16 (n = 10) or UIC2 (n = 3), anti-cadherin 5 mAb hec 1 (n = 5), anti-CD31 mAb hec 7 (n =3), anti-MHC I mAb W6/32 (n = 3), anti-CD18 mAb IB4 (n = 2), verapamil (n =3), or the vehicle control for verapamil,

20 0.03% methanol (Vehicle, n = 1). After three days of incubation, DC and T lymphocytes that appeared in the culture medium were collected and counted as previously described (7). Each experiment was conducted in triplicate; n = number of experiments in which condition was

included. The number of emigrated cells recovered from individual control explants was typically 5×10^5. To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each experiment was set equal to 1.0 and relative values were obtained for the remaining data.

5 **Figure 3 A-C.** Expression of MDR-1 in situ by DC. **(A)** Epidermis derived from explants cultured with anti-MDR-1 mAb MRK16 were fixed and Cy3-conjugated anti-mouse IgG was added to detect expression of MDR-1 (red). Addition of Cy3-labeled detection antibody to skin incubated in the presence of nonbinding control mAb (Hec1) showed no staining (not shown). **(B)** Immunostaining of same sample using FITC-conjugated anti-MHC II mAb

10 (green) identified dendritic cells. **(C)** Doubly-exposed frame to examine co-localization of p-glycoprotein and MHC II on dendritic cells (yellow). Bar is 10 μm.

Figure 4 A-E. Expression of functional MDR-1 by emigrated DC. **(A-C)** MDR-1 expression in emigrated skin cells was examined by flow cytometry using double-staining with anti-MDR-1 mAb and the DC lineage marker MHC II **(A, B)** or the T lymphocyte lineage

15 marker CD3 **(C)**. Quadrants were marked based on the level of fluorescence intensity observed in samples stained with negative control mAbs. **(D)** For studies measuring efflux, emigrated cells were loaded with the dye 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$) (- - -, no dye added) and cultured at 37 °C for 90 minutes without addition of mAb (filled profile), in the presence of UIC2 mAb (bold line), or kept at 4 °C for this duration (thin line).

20 Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II$^+$ cells. **(E)** Membranes prepared from purified, emigrated DC, PBMC, or HUVEC were

immunoblotted with anti-MDR-1 mAb C219. Numbers to the left of bands are molecular

weight markers.

Figure 5 A-F. Effect of verapamil on the efflux of the p-glycoprotein synthetic substrate

3,3'-diethyloxacarbocyanine iodide (DiOC$_2$) from dendritic cells. Panels A and B show the

5   fluorescence intensity of dendritic cells before loading with DiOC$_2$. In these cells, the FL1

staining intensity (x-axis) is low. Panels C-F show dendritic cells incubated under various

conditions after loading with DiOC$_2$, which fluoresces on the FL1 channel. Panels B-F show

cells after labeling for the cell surface marker MHC II (FL2 channel). Cell that shift 10^3

staining intensity on the FL2 channel are dendritic cells. For efflux measurements, dendritic

10   cells emigrated from skin were loaded with the dye and analyzed immediately for dye content

(time 0, Panel C), or cultured for 90 minutes at 37°C without additives (D) or in the presence

of verapamil (10 µg/ml) (Panel E) or the anti-p-glycoprotein mAb UIC2 (10 µg/ml) (Panel

F).

Figure 6 A-D. Retention of DC in the epidermis after treatment with MDR-1 antagonists.

15   (A) Epidermal sheets were stained with anti-MHC II mAb to enumerate DC before the onset

of culture or after three days of culture in the absence of mAb (no mAb, n = 7), or in the

presence of anti-cadherin 5 mAb hec 1 (n = 3); anti-MHC I mAb W6/32 (n = 3); anti-CD31

mAb hec 7 (n = 3); anti-MDR-1 mAb MRK16 (n = 6); anti-MDR-1 mAb UIC2 (n = 1);

verapamil (n = 2); or the vehicle control for verapamil, 0.03% methanol (Vehicle, n = 1). n =

20   number of experiments in which each condition was examined. DC were counted from *en*

face examinations of epidermal sheets in 16 to 20 high-power fields per experiment. Percent

reduction in DC density was calculated by comparing the number of DC in cultured explants

to the mean number present in a portion of the same skin sample before culture (typically 75

cells/field). **B-D**) Photomicrographs show the distribution of DC within the epidermis before

culturing of explants (**B**), after three days of culture under control conditions (no mAb) (**C**),

and after three days of culture in the presence of MRK16 (**D**). Bar is 20 μm.

5   **Figure 7**. Effect of anti-MDR-1 mAb on the maturation of DC. The levels of MHC II

expressed by epidermal DC were analyzed in gated epidermal suspensions by flow cytometry

before the onset of culture (Day 0) or after three days of culture in the absence of mAb or in

the presence of MRK16. Single cell suspensions of the epidermis were prepared by digestion

with dispase followed by trypsin. Keratinocytes and other skin cells were excluded from the

10   analysis by setting a gate to include only MHC II$^+$ cells.

Figure 8. Effect of anti-tissue factor monoclonal antibody (mAb) on emigration of dendritic

cells (DC) and T lymphocytes from skin explants. Explants of human skin were floated in

culture medium without added mAb or in medium containing anti-tissue factor mAb VIC7 or

control mAbs against cadherin 5 or carcinoembryonic antigen (CEA). After three days of

15   incubation, DC and T lymphocytes that appeared in the culture medium were collected.

Cumulative results from three independent experiments are shown. Each experiment was

conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered

from individual control explants were typically 5×10^5. To compare data from different

experiments, the mean number of emigrated cells in the absence of added mAb in each

20   experiment was set equal to 1.0 and relative values were obtained for the remaining data.

Figure 9 A-B. Analysis of expression of p-glycoprotein-related transporters in mice. A) Dendritic cells emigrated from wild-type or MRP knock-out mouse ear explants were loaded with the fluorescent transporter substrate Fluo-3 and cultured at 37° C for 60 minutes (open profile) or kept at 4° C for this duration (filled profile). Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II$^+$ cells. B) MRP expression in emigrated skin cells was examined by flow cytometry in permeabilized cells using double-staining with anti-MRP mAb MRPr1 (Signet Laboratories) or rat control mAb R35-95 (Pharmingen), followed by FITC-conjugated anti-rat mAb for detection.

Figure 10 A-B. Effect of MRP antagonist MK 571 on emigration of DC from skin explants. Explants of human skin or explants of the dorsal aspect of mouse ear were floated in culture medium with or without addition of MK 571 (25 μM). Murine emigrated dendritic cells were collected and explants were transferred to fresh medium daily (with or without MK 571) and were analyzed over 5 days. Human skin explants were analyzed on day 2, with a medium change or fresh addition of drug at day 1. Each experiment was conducted using 3 (human) to 10 (mouse) replicates and was performed on at least two independent occasions.

Figure 11 A-B. Contact sensitization in MRP knock-out mice or after administration of MRP antagonist to wild-type mice. A) Twenty five microliters of FITC dissolved in acetone:dibutylphthalate (8 mg/ml stock) was applied to the dorsothoracic area of shaved mice. Twenty four hours later, draining lymph nodes were collected, disaggregated to

obtain single cell suspensions, and stained for the dendritic cell marker CD11c. CD11c

was detected using biotinylated anti-CD11c and streptavidin-conjugated R-phycoerythrin.

Data are from a single experiment performed in quadruplicate. B). Mice were injected

with 1 mg MK 571 intraperitoneally before application of FITC and analysis as described

5   in (A). Data are from 2 experiments, each performed with triplicate samples.

DETAILED DESCRIPTION OF THE INVENTION

The following terms shall have the definitions set out herein. The terms "immunity" or

"development of immunity" are used generally to refer to the initial encounter of the immune

system with an antigen and the development of a specific immune response to the antigen.

10   This entails, in brief, the uptake of the antigen by antigen-presenting cells (APCs), especially

dendritic cells (DC), processing of the antigen, and presentation of antigen peptides in a

complex with MHC molecules, to T lymphocytes, with the subsequent formation of antigen-

specific T lymphocytes, including memory, helper, and cytotoxic T lymphocytes, as well as

antibody-producing B lymphocytes. The term immune response is generally used herein to

15   refer to the response of the immune system to a subsequent encounter with an antigen to

which immunity has been previously established. One aspect of this encounter is the uptake

of the antigen by APCs, migration to draining lymphoid organs including lymph nodes and

the spleen, and subsequent presentation to memory T lymphocytes that circulate in lymphoid

tissues.

20   The terms "inflammation" and "inflammatory response" refer to the acute or chronic response

to a known or unknown antigen in which a particular general or local site within the body

exhibits adverse sequelae of the accumulation of white blood cells, including monocytes, and

their secreted mediators, producing a range of effects of varied intensities including pain,

tenderness, edema, destruction of tissue, organ failure, asthma, and urticaria (hives). In the

case of atherosclerosis, a chronic inflammatory disorder of the arterial wall, accumulation and

retention of monocytes in the intimal tissue of the artery leads to lipid-laden plaque formation

5   and danger of thrombosis.

As described above, the present invention relates to methods and agents for the modulation of

the immune response involving the dendritic cell membrane proteins p-glycoprotein and

tissue factor. As will be seen in the examples below, these proteins have been discovered to

have an important physiological role in the migration of dendritic cells from the peripheral

10   tissues, a location where antigen is encountered, to the lymphatic vessels, where interaction

of the dendritic cell with T lymphocytes induces immunity or an immune response.

Manipulation of these proteins to antagonize or agonize their activities has been found to be

useful for decreasing or increasing, respectively, the ability of the dendritic cell to migrate to

the lymphatic vessels and thus modulate the development of immunity or an immune

15   response to an antigen or antigens encountered by dendritic cells.

p-Glycoprotein, also known as MDR-1, is a large plasma membrane protein that functions as

an ATP-driven chemotherapeutic drug efflux pump. The endogenous substrate(s) for the p-

glycoprotein transporter has not yet been identified, but based on structure-activity

relationships of numerous compounds, are believed to include cationic, hydrophobic

20   molecules with at least two planar rings and a molecular weight of 400-1500 (40).

Endogenous substrates also likely include modified phospholipids, such as phospholipids that

are involved in cell signaling. The presence of p-glycoprotein on cells confers multi-drug

resistance ("MDR"); in cancer cells, p-glycoprotein pumps out of the cell chemotherapeutic

agents and thus renders cancer cells resistant to chemotherapy (36). Thus, to enable or

improve cancer chemotherapy in resistant tumors, p-glycoprotein has been a target for

pharmacologic intervention to disable the pump (36). Numerous compounds have been

5   found which antagonize the activity of the pump, and several are in use or in clinical

development as adjunct therapy with cancer chemotherapeutic agents to block resistance.

Prophylactic use has also been suggested to prevent the appearance of a drug resistant

phenotype.

Several gene products that are highly homologous to p-glycoprotein also confer drug

10   resistance and can transport a similar profile of substrates as p-glycoprotein. Examples of

these related proteins include cMOAT (canalicular multiple organic anion transporter), LRP

(lung resistance protein), and MRP (multi-drug resistance related protein). Expression of

these p-glycoprotein-related gene products in monocytes or dendritic cells of humans or other

mammalian species may confer the same function as the MDR-1 gene product. Therefore,

15   p-glycoprotein as defined herein embraces the MDR-1 gene product or the gene product(s) of

structurally and functionally related transporters that mediate dendritic cell migration in

humans and other mammals.

Methods and agents which have been found to effectively antagonize the activity of the p-

glycoprotein protein are useful in the practice of the present invention for antagonizing p-

20   glycoprotein and thus inhibiting dendritic cell migration and suppressing immunity or an

immune response. These methods and agents comprise several approaches, including but not

limited to antibodies and fragments which bind to p-glycoprotein, compounds which reverse

multi-drug resistance, and means to reduce the expression of p-glycoprotein. These methods

and agents may be used singly or in combination. As will be described in more detail below,

administration may be targeted to a particular location of the body where the activity is

desired, for example on or in an cutaneous lesion such as psoriasis or contact dermatitis, and

5   delivery by aerosol or powder to the bronchi or lungs in the case of asthma.

Suppression or down-regulation of immunity and the immune response has utility in the

prophylaxis and treatment of numerous diseases and conditions. Methods and agents of the

present invention are useful as prophylaxis against the exposure of an individual to a known

immunogen or allergen against which an immune response would be highly undesirable. For

10   example, acute exposure to a causative agent of contact dermatitis, such as might occur in an

industrial setting, for example, exposure to protein-modifying metals and their salts such as

nickel and chromate as a result of a chemical spill, or release of a gaseous or airborne

particulate allergen; a further example is topical or pulmonary exposure to urushiol, from

contact with poison ivy or inhalation of smoke from burning foliage. Immunity to accidental

15   oral consumption of a potent immunogen may be blocked by the methods and agents of the

present invention. An individual exposed under such conditions can prevent the delivery of

the antigen to the lymph nodes by the methods and agents of the present invention.

Wider utility of the methods and agents of the present invention are found in the treatment of

acute and chronic conditions mediated by the initial or repeat exposure of the immune system

20   to a known or as-yet unidentified antigen causative of a condition or disease. A role for

dendritic cells in the development of immunity has been established; the role of dendritic

cells in the subsequent immune response has been established for many antigens and is

suspected in others. The agents of the present invention that suppress the migration of

dendritic cells are useful for one or both of these aspects, and especially in the type of

immune response known as delayed-type hypersensitivity and more particularly, contact

hypersensitivity. Examples of known immunogens that cause disease include pollen and

5   other airborne allergens which cause allergic rhinitis and other seasonal allergic reactions,

hay fever, allergic conjunctivitis; arthropod-borne antigens such as dist mites and insect

secretions such as those that cause the reaction to mosquito bites; foods that cause allergies,

etc. In numerous autoimmune diseases, suspected antigens to which an immune response is

responsible for the pathology have been identified; in others, the antigens are probable, and in

10   others, they are uncertain. Examples of as-yet identified immunogens responsible for

pathological inflammatory conditions include food allergies of unknown cause, irritable

bowel syndrome, rheumatoid arthritis, psoriasis and a large number of autoimmune diseases

which are believed to be initiated and maintained by endogenous antigens. In a further utility

of the present invention, means to block an immune response to transplanted antigens, in the

15   form of acute rejection, is provided. This may be used as an adjunct to immunosuppressive

and other agents routinely used in transplantation.

The methods and agents of the present invention are not limited to, but are particularly useful

in, the treatment of inflammatory conditions where the acute or chronic exposure to antigen

occurs in the periphery, such as the skin, as well as the gut and the lungs.

20   As mentioned above, one particular condition amendable to treatment by the methods and

agents of the present invention is in the area of transplantation. A critical role has been

identified in the interaction of antigen-presenting cells, dendritic cells in particular, with T

lymphocytes in initiating rejection. It is well established that dendritic cells mediate allograft

rejection, since experimental techniques used to deplete allografts of endogenous dendritic

cells do not give rise to subsequent rejection responses in recipients. However, it is not

clinically feasible to deplete allografts of endogenous dendritic cells. Because it is the

5   migration of dendritic cells to the graft recipient's lymph nodes and spleen that ultimately

results in graft rejection, clinically feasible methods to block dendritic cell migration to these

lymphoid tissues would prevent graft rejection. Suppression of the migration of dendritic

cells by agents which antagonize p-glycoprotein or tissue factor and thus inhibit their

interaction with T lymphocytes blocks the development of the immune response and thus are

10   useful for averting graft rejection.

Another disease particularly amenable to both prophylaxis treatment by the methods and

agents of the present invention is HIV. Transepithelial infection by HIV is believed to be

mediated by dendritic cells, which become infected with the virus and carry the virus to

lymph nodes where interactions between dendritic cells and T lymphocytes as described

15   above lead to infection of T lymphocytes. It is known that bone marrow-derived dendritic

cells facilitate the creation of a population of CD 4+ T lymphocytes, which are a major

reservoir of HIV infection. Methods and agents of the present invention which block the

migration of dendritic cells from peripheral sites, where dendritic cell HIV infection occurs,

can interrupt the endogenous spread of infection; suppressing further dendritic cell migration

20   inhibits the dendritic cell-mediated fostering of infected T lymphocyte populations.

In one embodiment of the present invention, antibodies or antibody fragments to p-

glycoprotein may be administered to inhibit dendritic cell migration. Antibodies may be

made which bind to the extracellular portion of the p-glycoprotein molecule. Non-limiting

examples of suitable antibodies include monoclonal antibodies MRK16 (24), 4E3 (23), and

UIC2 (22). Such antibodies include but are not limited to polyclonal, monoclonal, chimeric,

single chain, Fab fragments, and an Fab expression library. Other fragments of such

5   antibodies capable of binding to an epitope on the p-glycoprotein molecule are embraced

herein. The anti-p-glycoprotein antibodies of the invention may be cross reactive, e.g., they

may recognize p-glycoprotein from different species. Polyclonal antibodies have greater

likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for

a single form of p-glycoprotein, such as murine p-glycoprotein. Preferably, such an antibody

10   is specific for human p-glycoprotein.

Various procedures known in the art may be used for the production of polyclonal antibodies

to p-glycoprotein. For preparation of monoclonal antibodies directed toward p-glycoprotein,

or fragment thereof, any technique that provides for the production of antibody molecules by

continuous cell lines in culture may be used. These include but are not limited to the

15   hybridoma technique originally developed by Kohler and Milstein [*Nature* **256**:495-497

(1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et

al., *Immunology Today* **4**:72 1983); Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* **80**:2026-2030

(1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole

et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)].

20   In an additional embodiment of the invention, monoclonal antibodies can be produced in

germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the

invention, techniques developed for the production of "chimeric antibodies" [Morrison et al.,

J. Bacteriol. **159**:870 (1984); Neuberger et al., *Nature* **312**:604-608 (1984); Takeda et al.,

Nature 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific

for p-glycoprotein together with genes from a human antibody molecule of appropriate

biological activity can be used; such antibodies are within the scope of this invention. Such

human or humanized chimeric antibodies are preferred for use in therapy of human diseases

5   or disorders (described *infra*), since the human or humanized antibodies are much less likely

than xenogenic antibodies to induce an immune response, in particular an allergic response,

themselves.

According to the invention, techniques described for the production of single chain antibodies

[U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted

10   to produce p-glycoprotein-specific single chain antibodies. An additional embodiment of the

invention utilizes the techniques described for the construction of Fab expression libraries

[Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of

monoclonal Fab fragments with the desired specificity for p-glycoprotein.

In a second, and preferred, embodiment of the present invention, administration of agents to

15   inhibit dendritic cell migration are provided which are known to reverse multiple drug

resistance. Suitable agents may be selected from compounds that inhibit this drug

transporter, including such non-limiting examples as calcium channel blockers such as

verapamil, diltiazem, dihydroperidine, nifedipine, bepridil, and nicardipine; other

cardiovascular drugs, including amiodarone, dipyridamole, and quinidine; antimalarial

20   compounds such as quinine, quinacrine, and cinchonine; cephalosporin and other antibiotics

such as cefoperazone, ceftriaxone, erythromycin, valinomycin, bafilomycin, and gramicidin;

cyclosporins such as cyclosporine A and staurosporine; phenothiazines such as

trifluoperazine, chlorpromazine and fluphenazine; hormones such as tamoxifen,

N-desmethylramoxifen, 4-hydroxytamoxifen, toremifene, and progesterone, and other

compounds such as terfenadine, monensin, yohimbine, reserpine, cremophor, benzquinamide,

estramustine, and tacrolimus.

5   In a preferred embodiment, more potent inhibitors of multi-drug resistance than those

described above are used in the practice of the present invention. Non-limiting examples

include the cyclosporine analog PSC 833, MK 571, and the calcium channel blockers

dexverapamil and dexniguldipine. PSC 833 is a cyclosporine analog with greater binding

specificity for p-glycoprotein (36); it is 10-fold more potent than cyclosporine A and can

10   reverse multi-drug resistance in vitro at concentrations of 0.5 to 2.0 μmol/L. MK 571 is a

substance that antagonizes p-glycoprotein and murine multidrug resistance related protein

(MRP), and is a particularly potent inhibitor of the latter. MK 571 is known to be an

antagonist of the leukotriene D_4 receptor (54), and as will be seen in the Examples below, has

utility for the purposes described herein. Other potent inhibitors of p-glycoprotein include

15   S9788, rapamycin, MK 571, GF120918, and SR33557.

In a further embodiment, inhibition of p-glycoprotein in dendritic cells may be achieved by

targeting gene expression with antisense oligonucleotides. This approach has been

demonstrated feasible in vitro using mouse 3T3 fibroblasts (41).

As described above, another dendritic cell surface protein that may be antagonized to achieve

20   the practice of the present invention is tissue factor (abbreviated TF). Tissue factor is a

procoagulant protein present on the surface of numerous cell types, including endothelial

cells, which line the vasculature, and monocytes, which circulate in blood. Increased

expression of tissue factor by these cell types has been linked to many thrombotic disorders

and pathologic states (see below with regard to increased expression). The majority of

monocyte membrane-associated tissue factor is not in an enzymatically active form that can

5   initiate clotting; in order to become active, tissue factor must form an active complex with

another of the clotting factors, Factor VII or its activated form, Factor VIIa. This complex

then leads to clot formation.

Also as described above in the instance of antagonists of p-glycoprotein, tissue factor may

also be antagonized in order to inhibit dendritic cell mobility. This may be achieved by the

10   use of antibodies and fragments against tissue factor. A monoclonal antibody against tissue

factor that inhibits migration of DC is VIC7. Compounds known to inhibit tissue factor

activity include the dilazep (42) and retinoic acid (43). In a further embodiment, inhibition of

tissue factor expression in dendritic cells may be achieved by targeting gene expression with

antisense oligonucleotides to tissue factor (49). A further strategy on interfering with tissue

15   factor activity in order to suppress dendritic cell migration is through the use of purified,

nonlipidated fragments of soluble recombinant human TF. Migration was inhibited in the

presence of a soluble, recombinant form of human TF representing the complete amino acid

sequence of the extracellular region or fragments thereof, expressed in E. coli and used in

delipidated form. Soluble TF impeded migration by $69 \pm 2\%$ in eight independent

20   experiments when used at 5-20 µg/ml. Only fragments containing amino acid residues that

are carboxyl to residue 202 blocked reverse transmigration effectively. This finding agrees

well with the location of the epitope for mAb VIC7, which is between amino acids 181-214.

The finding that both anti-TF mAb and soluble TF inhibit reverse transmigration is consistent

with a model in which the binding of endogenous TF on monocytes to an essential ligand is

prevented by anti-TF mAb and is competed with by soluble recombinant TF. The

competitive binding sites contained in the region spanning 202-219.

5   Enhancing the effectiveness of vaccination and other forms of immunotherapy is another

feature of the present invention. This aspect is carried out using methods and agents which

enhance or activate the dendritic cell membrane proteins p-glycoprotein and tissue factor.

Agents useful for this utility include activators of p-glycoprotein, such as bromocriptine (44),

N-acetyl-leucine$_n$-tyrosine amide where n=1-6 (45), and cytarabine (46) are preferred.

10   Suitable activators of tissue factor include endotoxin (47), silver ion (47), and N,N'-dimethyl-

γ,γ'-dipyridylium dichloride (48). Preferably, these agents are admixed with the immunogen

to be used for vaccination.

A further utility of one aspect of the present invention is in the immunotherapeutic treatment

of cancer. Numerous studies and trials involve attempts to initiate or enhance an immune

15   response in the patient toward the patient's own tumor. However, these regimens have

provided little positive results, often because the patient is unable to mount an effective

immune response to the tumor antigens. In this case, effective antigen presentation maybe

suppressed. The methods and agents of the present invention directed towards the

enhancement of the immune response and increasing the effectiveness of vaccines has

20   particular utility in the enhancement of therapeutic vaccines for cancer.

In another embodiment of the present invention, methods and agents may be used to increase dendritic cell migration in order to achieve an enhancement of the development of immunity or in a subsequent immune response to an antigen. This would be carried out using agents that increase transport of endogenous substrates for p-glycoprotein that mediate dendritic cell

5   migration. Enhanced development of immunity is particularly useful in enhancing the effectiveness of a vaccine, such as that for viruses such as poliomyelitis, rubella, measles, variola, and varicella, and especially poorly immunogenic immunogens such as influenza; bacteria such as *Borrelia burgdorferi*, the causative pathogen of Lyme disease, and other organisms including parasites such as malaria. Furthermore, the invention herein is also

10   useful in the various newer immunotherapeutic approaches to the prevention and control of other infectious diseases such as HIV and hepatitis, and immunotherapies of various cancers (infra). In these approaches, potentially immunogenic components of the virion or tumor are prepared by various means and introduced into the patient in an attempt to develop immune recognition of the pathologic antigen(s). Often, the patient is immunocompromised and

15   cannot mount an effective immune response to the antigen. One embodiment of the methods and agents of the present invention provide a means for increasing the delivery of antigen to the immune system by increasing or enhancing the migration of dendritic cells which have processed the antigen to the lymphatic vessels, where they may subsequently encounter T lymphocytes and induce cellular and humoral immunity.

20   The route of delivery of the agents of the present invention may be selected by one skilled in the art to provide the optimal contact with dendritic cells involved in the etiology of the condition to be treated or the route of exposure of the immunogen. As examples, contact dermatitis, psoriasis, and other topical conditions may be treated by application of the agent

of the present invention to the skin. Allergic conjunctivitis may be treated by application of

the agent to the eye. Parenteral or other routes of administration which results in generalized

delivery of the agent is also provided. Bronchiolar and pulmonary exposure may be achieved

by aerosol and fine powder inhalation, respectively.

5   In yet another aspect of the present invention, provided herein are pharmaceutical

compositions of the above agents. Such pharmaceutical compositions may be for

administration for injection, or for oral, pulmonary, nasal or other forms of administration. In

general, comprehended by the invention are pharmaceutical compositions comprising

effective amounts of a low molecular weight component or components, or derivative

10   products, of the invention together with pharmaceutically acceptable diluents, preservatives,

solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of

various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives

such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants

(*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol)

15   and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate

preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into

liposomes. Hylauronic acid may also be used. Such compositions may influence the physical

state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and

derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack

20   Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by

reference. The compositions may be prepared in liquid form, or may be in dried powder,

such as lyophilized form.

Contemplated for use herein are oral solid dosage forms, which are described generally in

Remington's Pharmaceutical Sciences, 18th Ed.1990 (Mack Publishing Co. Easton PA

18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include

tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid

5   encapsulation may be used to formulate the present compositions (as, for example, proteinoid

microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used

and the liposomes may be derivatized with various polymers (*e.g.*, U.S. Patent No.

5,013,556). A description of possible solid dosage forms for the therapeutic is given by

Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10,

10   1979, herein incorporated by reference. In general, the formulation will include the

component or components (or chemically modified forms thereof) and inert ingredients

which allow for protection against the stomach environment, and release of the biologically

active material in the intestine.

In a further embodiment of the present invention, methods and agents are provided for the

15   augmentation of the migration of monocytes, by enhancing the functioning of the monocyte

membrane protein p-glycoprotein. The selection of suitable agents and routes of delivery are

as those described above in relation to modulating the same membrane protein on dendritic

cells. This aspect of the invention has utility in the treatment of various disorders, preferably

chronic inflammatory conditions which are not necessarily, but may be, initiated or

20   promulgated by antigens and antigen-specific immune cells such as cytotoxic T lymphocytes.

For example, the development and progression of atherosclerotic lesions involves the

progressive accumulation and retention of monocytes (which become tissue macrophages)

and other cell types in lesions; the local inflammation mediated by their secretory products

which attracts more pro-inflammatory cells, together with secretion of growth factors, is a

pathophysiologic basis for vessel occlusion.

The methods and agents of the present invention directed to increasing monocyte migration

has particular utility in preventing the retention of monocytes in inflammatory sites within the

5   body, thus limiting the extent of inflammation. Other examples of diseases and condition in

which accumulation and retention of monocytes and resultant production of inflammatory

mediators, growth factors and chemoattractants for additional inflammatory cells include

rheumatoid arthritis and the granulomatous diseases.

It is a further object of the present invention to provide methods for identifying agents and

10   compound useful in modulating the migration of dendritic cells. As a consequence of the

discovery by the inventors herein of the presence of p-glycoprotein on the membrane of

dendritic cells and the further discovery of the role of p-glycoprotein and tissue factor in

dendritic cell migration, various known methods for measuring the effect of compounds on p-

glycoprotein activity may be adapted for use on dendritic cells. For example, dendritic cells

15   may be isolated from skin explants and loaded with a dye known to be exported by

functioning p-glycoprotein (28), such as 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$). Export

of the dye may be measured in the absence and presence of compounds suspected of

inhibiting or enhancing the activity of p-glycoprotein by decreasing or increasing,

respectively, the pumping of the dye out of the cell. In a further embodiment, agents useful

20   for the modulation of dendritic cell migration may be identified by first identifying whether a

candidate agent is capable of interacting with p-glycoprotein or tissue factor; and

subsequently determining the extent of modulation of the agent on the migration of dendritic

cells in vitro or in vivo. The initial screen may be performed on cells which express p-glycoprotein, such as any one of numerous cancer cell lines.

Subsequently, the effect of the compound on the migration of dendritic cells may be performed as described below or that described in U.S. Patent 5,627,025, incorporated herein
5   by reference. This method employs human skin explants in culture to measure the effect of compounds on dendritic cell emigrating from the skin explant into the culture medium. The methods disclosed herein are not limited to any particular method but are based upon the newly-identified role of p-glycoprotein and tissue factor on dendritic cells in cell migration.

Likewise, assays may be performed to identify compounds which enhance migration of
10 monocytes out of tissues. As one non-limiting example, migratory behavior of monocytes may be evaluated in an in vitro model of the blood vessel wall consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels (see Example I, below). Human blood monocytes, which express p-glycoprotein, transmigrate across the confluent endothelium and enter the collagenous substrate. The extent of migration is measured after
15 exposure of the monocytes to the candidate. Other methods of measuring the migration of monocytes are known to the skilled artisan and are useful in the practice of the present invention.

In a further example of a method for identifying compounds useful for modulating dendritic cell p-glycoprotein activity, the following steps are carried out:
20   i)   loading isolated dendritic cells with a detectable substrate of the p-glycoprotein transporter activity;

ii)   exposing the loaded cells to an agent suspected of modulating p-

glycoprotein activity;

iii)   measuring the rate of transport of the detectable substrate from the

dendritic cells; and

5   iv)   comparing said rate to the rate of p-glycoprotein transporter activity

determined of control dendritic cells not exposed to the agent.

An increase or decreased in the transport rate in the presence of the agent indicates the extent

of activity of the agent in increasing or decreasing, respectively, p-glycoprotein activity of

said dendritic cells. In a further step, compounds that are active in the above assay, either at

10   increasing or decreasing p-glycoprotein activity, can be additionally assays to determine if

they are useful for modulating the migration of dendritic cells by determining the extent of

modulation of the agent of the migration of dendritic cells in vitro or in vivo. DC migration

may be determined by any of several techniques, such as the emigration rate described in

aforementioned U.S. Patent 5,627,025. In another method, compounds may be evaluated

15   directly in the emigration assay (7). Activity may be evaluated in vivo by administration of

the agent to an animal, followed by, for example, application of an antigen to the skin which

would under normal circumstances induce immunity in the animal or an immune response.

The extent of the any reduction in the immune response elicited after administration of the

agent is an indication of the extent of inhibition of dendritic cell migration. Similarly,

20   compounds to be evaluated for the enhancement of the immune response may be evaluated in

vivo by administering the agent parenterally or to the skin, for example, followed by

application of an immunogen. Any resulting enhancement of the immune response as

compared with control animals indicates positive activity in enhancing the immune response.

The following examples are presented in order to more fully illustrate the preferred

embodiments of the invention. They should in no way be construed, however, as limiting the

broad scope of the invention.

MATERIALS AND METHODS

5   *Transendothelial Migration Assays* Using a method detailed previously (10, 11), freshly

isolated peripheral blood mononuclear cells (PBMC), approximately one-fourth of which are

monocytes, were added to confluent monolayers of unstimulated HUVEC grown on type I

bovine collagen gels with or without addition of mAb. Cultures were incubated for 1.5 hr;

during this time, monocytes, but very few lymphocytes, transmigrate into the collagen (10).

10   To examine their subsequent basal-to-apical transendothelial migration, monocytes were

allowed to accumulate beneath the endothelium in the absence of added mAb. Then

monolayers were washed twice in Medium 199 (Life Technologies, Grand Island, NY) to

remove nonadherent cells from the apical surface, and individual wells received aliquots of

Medium 199 containing 20% heat-inactivated human serum with or without added

15   monoclonal antibodies (mAbs) as indicated. After 24 hr of incubation, cultures were washed

twice in Medium 199 and control medium or medium containing mAb was replenished.

After a total time of 48 hr in culture, samples were fixed for microscopic analysis.

Differential interference contrast optics were used to quantitate the number of mononuclear

phagocytes (MP) that were beneath the endothelial monolayer (11) in at least five high-power

20   fields per sample. Each experiment was conducted with six replicates per parameter tested.

Skin Cultures Human split-thickness skin was obtained from the New York Firefighter's Skin

Bank (New York Hospital-Cornell Medical Center) from cadavers within 24 hr of death, or

from patients undergoing plastic surgery, and was authorized for use in research. Generally,

dermatomes were approximately 300-mm thick, including both epidermis (about 100-mm)

and a portion of the dermis. Skin was prepared, cultured, and emigrated cells were

quantitated as previously described (7). Each explant was trimmed to 400-mm^2 and floated in

5   3 to 6 ml culture medium. When used, mAbs or verapamil (Sigma) were added to the culture

medium at the onset of culture, and cultures were incubated undisturbed until the indicated

day of analysis. Verapamil was prepared as a concentrated stock in methanol. Final

methanol concentration in cultures was 0.03% (vol/vol).

Immunostaining and Flow Cytometry Skin explants were separated into epidermal and

10   dermal sheets by treatment with 0.5 M ammonium thiocyanate for 20 minutes at 37° C (12).

Alternatively, skin samples were snap-frozen in OCT compound (Miles; Elkhart, IN) and

then used for the preparation of thick sections (approximately 100 mm) cut parallel to the

epidermis (6). After either procedure, sheets of skin were fixed in acetone for 5 min. before

staining. Sheets of epidermis or thick sections were incubated overnight at 4°C in Eppendorf

15   tubes with CY3-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch

Laboratories, Inc.),1:20 FITC-conjugated anti-MHC II (Becton Dickinson), or anti-MHC II

9.3C9 hybridoma supernatant. Tissues were washed three times in PBS containing 1%

Tween 20, 15 min. each at room temperature. For detection of 9.3C9 when it was used,

CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Inc.) was added for 2 hr at

20   4° C, followed by three washes as just described. Staining for flow cytometry was carried out

as previously detailed (7).

In some experiments, explants were incubated in dispase II (Boehringer Mannheim) for 45

min. at 37°C to separate the epidermis from dermis. Pieces of epidermis were submerged in

0.05% trypsin/0.53 mM EDTA for 45 min. Single cell suspensions were generated by gently

pressing epidermal pieces through a fine mesh. Aliquots of digested cells were incubated

5   with FITC-labeled anti-MHC II for 30 min. on ice, washed twice, and analyzed by flow

cytometry.

Immunoblots Emigrated DC were purified by negative selection: anti-CD3 mAb was

incubated with emigrated cells, and T lymphocytes were removed using anti-mouse

IgG-conjugated magnetic beads (Dynal). The purity of the resulting population was verified

10   by flow cytometric evaluation after staining with PE-conjugated anti-MHC II (Becton

Dickinson). Microsomal membranes from 5 million thus purified DC, 25 million PBMC, or

5 million HUVEC were prepared as described (13), subjected to SDS-PAGE (4-12% gradient

gel) under reducing conditions, and electroblotted onto a nitrocellulose membrane. The

membrane was probed with 5 mg/ml C219 anti-MDR-1 mAb (Centocor) and visualized using

15   peroxidase-conjugated anti-mouse IgG, followed by ECL substrate (Amersham).

Efflux Assays A previously published method to detect functional MDR-1 (14) was

employed with minor modifications. Emigrated cells were incubated in RPMI-1640

containing 20 ng/ml $DiOC_2$ (Molecular Probes) for 15 min. at 37 °C, washed twice in ice cold

medium, resuspended in 10% fetal bovine serum/RPMI-1640 containing no additives or with

20   addition of 10 mg/ml UIC2 mAb (Coulter). After 90 min. of incubation at 37 °C, cells were

washed and immediately placed on ice for labeling with PE-conjugated anti-MHC II mAb

and analysis by flow cytometry.

EXAMPLE 1:   **Role of MDR-1 in basal-to-apical transendothelial migration of**

mononuclear phagocytes.

The present inventors were drawn to investigate the role of p-glycoprotein in dendritic cell

migration by a set of observations with blood monocytes, now known to be a progenitor of

5   DC when appropriately stimulated with cytokines (15-19). In these initial experiments, the

migratory behavior of monocytes was examined in an in vitro model of the blood vessel wall

consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels

(10, 11). Human blood monocytes, which express MDR-1 (20, and data not shown),

transmigrate across the confluent endothelium and enter the collagenous substrate. This

10   initial migration into the collagen, which is completed within one hr, was unaffected by the

addition of anti-MDR-1 mAb, but was partially blocked by mAbs against the β_2 chain of

integrins (CD18) and CD31, as described (10, 11) (**Fig. 1A**).

After a brief residence in the collagen, a majority of subendothelial MP retraverse the

overlying, intact endothelium with a $t_{1/2}$ of 24 to 48 hr (21). This reverse transendothelial

15   migration was not prevented by mAb to CD18 or CD31 (**Fig. 1B**). However, screening of

mAbs (> 100) submitted to the VIth International Workshop for Human Leukocyte

Differentiation Antigens revealed that neutralizing mAbs to MDR-1 strongly inhibited

reverse transmigration. UIC2 (22), 4E3 (23), and MRK16 (24) against extracellular domains

of MDR-1 inhibited reverse transmigration by 50%, 64%, and 88%, respectively (**Fig. 1B**).

20   MRK16, used here as F(ab')$_2$ fragments, was the most potent blocking mAb, exhibiting

maximal levels of inhibition using as little as 0.5 to 1.0 mg/ml. C219 (25), which binds an

intracellular epitope of MDR-1, had no effect. Preincubation of monocytes with MRK16,

and subsequent removal of unbound mAb before addition of these cells to the endothelium

blocked reverse transmigration as well as when the mAb was present continuously during the

assay (Pre-MRK16, **Fig. 1B**). No other mAbs screened inhibited reverse transmigration,

except one against tissue factor.

5   **EXAMPLE 2:   Role of MDR-1 in mobilization of DC and T lymphocytes from**

skin.

During reverse transmigration, MP migrate across endothelium in a basal-to-apical direction,

a movement that is reminiscent of the normal trafficking of DC from the periphery into

afferent lymphatics. A panel of mAbs, including those against MDR-1, was applied to

10   cultured human skin explants. Appearance of DC and T lymphocytes in the culture medium

of the explants was reduced by 71 ± 9%, and 79 ± 16% when the skin was incubated in the

presence of MRK16 at concentrations of 2 and 10 mg/ml, respectively (**Fig. 2A**). Verapamil,

a drug that antagonizes MDR-1 transport (26), reduced DC and T lymphocyte accumulation

in the culture medium by 52 ± 19% at 10 mg/ml (20 mM) (**Fig. 2A**). The anti-MDR-1 mAb

15   UIC2 also blocked migration, by 75 ± 2% when used at 2 mg/ml (**Fig. 2A**). An isotype

matched control mAb against cadherin 5 (Hec 1) (11) that does not react with DC or T cells

had no effect. Moreover, isotype-matched mAbs against other DC surface proteins--CD18

(IB4), CD31 (hec7), and MHC I (W6/32)--also did not inhibit the migration significantly

(**Fig. 2B**). Under all conditions, the cellular content of émigrés ranged from 40 to 60% DC,

20   with the remaining population being T lymphocytes. Thus, antagonists of MDR-1 blocked

migration of DC and T lymphocyte uniformly. Cell viability in all groups was >90%, as

assessed by trypan blue exclusion.

EXAMPLE 3:   **Expression of MDR-1 by DC.**

T lymphocytes are known to express MDR-1 (14, 20, 27), but its expression by DC has not

been reported previously. Immunostaining for MDR-1 was observed on DC in the epidermis

(**Fig. 3A and C**). The same cells also stained positively for the DC marker MHC II (**Fig. 3B**

5   **and C**). No other cell types in the skin, such as keratinocytes or fibroblasts, showed positive

staining for MDR-1 in these experiments. For these studies, skin was cultured in the

presence of MRK16 for two days, as for migration analysis. Then staining was carried out on

tangential sections prepared from snap-frozen skin, applying only anti-mouse Cy3-conjugated

detection antibody. This approach illustrates not only the presence of MDR-1 on epidermal

10   DC in situ, but also demonstrates that mAb applied during culture gains sufficient access to

the epidermis. Skin incubated with isotype-matched control mAb hec 1 did not show positive

staining.

Flow cytometric analysis of the cells that emigrated from control explants using double

labeling with anti-MDR-1 (using either MRK16 or UIC2) and anti-MHC II mAbs

15   demonstrated high levels of MDR-1 on all DC in some experiments (**Fig. 4A**) or on a

majority, but not all, DC in other experiments (**Fig. 4B**). The fraction of DC staining

positively was always greater than the fraction of positively stained subset of T lymphocytes

(**Fig. 4C**).

Expression of authentic MDR-1 by DC was evident in two assays. To document expression

20   of functional MDR-1 (28), DC were loaded with a synthetic, fluorescent substrate for

MDR-1, $DiOC_2$. At 37 °C, but not 4 °C, DC transported this substrate into the surrounding

medium, showing a log decrease in fluorescence intensity (**Fig. 4D**), and this efflux was

-34-

inhibited by anti-MDR-1 mAb UIC2 (**Fig. 4D**). To identify MDR-1 molecules in DC, crude

membranes were prepared and immunoblotted using mAb C219, another mAb that reacts

with MDR-1. A broad band of approximately 170 to 180 kDa, the reported molecular weight

of MDR-1 was identified (**Fig. 4E**). The band observed matched that seen in membranes

5   from PBMC and was not observed in HUVEC (**Fig. 4E**), which do not express MDR-1.

EXAMPLE 4:   Effect of Verapamil on the DiOC$_2$ Efflux from Dendritic Cells

Figure 5 further documents that functional p-glycoprotein is expressed by dendritic cells and

that p-glycoprotein-mediated transport can be blocked by agents, particularly verapamil and a

monoclonal antibody against p-glycoprotein, that are known to inhibit p-glycoprotein

10   transport activity. As described in the Brief Description of **Figure 5**, dendritic cells were

loaded with the p-glycoprotein synthetic substrate 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$

), and flow cytometry performed as described in Example 3. Approximately 90% of the dye

was transported out of the dendritic cells incubated in the absence of p-glycoprotein

inhibitors, as indicated by the one-log decrease in fluorescence intensity in the FL1 channel

15   (x-axis) (compare Panel C to D). However, transport of the dye was completely prevented in

the presence of verapamil (Panel E) and the anti-p-glycoprotein monoclonal antibody UIC2

(Panel F).

Example 5:   Effect of MDR-1 antagonists on maturation and retention of DC in

epidermis.

20   To examine the step at which emigration of DC from the explants was inhibited by

anti-MDR-1 mAb, epidermal and dermal sheets prepared from cultured skin explants were

stained for MHC II (29, 30). Compared to the number present at the onset of culture (**Fig.**

6A, B), the number of DC remaining in the epidermis after three days of culture under control

conditions was decreased by 56% (**Fig. 6A, C**). In explants cultured in the presence of

MRK16 (**Fig. 6A, D**), UIC2 (**Fig. 6A**), or verapamil (**Fig. 6A**), a more limited reduction in

DC density of 13%, 11%, and 25%, respectively, were observed. Thus, antagonism of

5   MDR-1 results in retention of DC in the epidermis.

In cultures treated with anti-MDR-1 mAb MRK16, some DC retained in the epidermis

appeared morphologically similar to epidermal DC before the onset of culture (compare **Fig.**

6B and D), but others showed evidence of maturation resembling that observed under control

conditions (**Fig. 6C** and data not shown), including an increase in cell size and greater

10   intensity of staining for MHC II (4, 29, 30). Flow cytometric analysis of DC enzymatically

removed from the epidermis after culture in the presence of anti-MDR-1 mAb MRK16 or

under control conditions indicated that the maturation of DC, which usually accompanies

their mobilization, was not inhibited by anti-MDR-1, as exemplified by the up regulation of

MHC II in the majority of DC in the presence or absence of MRK16 (**Fig. 7**). Thus,

15   antagonism of MDR-1 strongly impedes the migration of DC, but only inconsistently or

negligibly suppresses their maturation.

EXAMPLE 6:   **Effect of Anti-Tissue Factor Antibodies on Emigration of**

                     Dendritic Cells and T Lymphocytes from Skin Explants

Explants of human skin were floated in culture medium without added monoclonal antibody

20   or in medium containing anti-tissue factor monoclonal antibody VIC7 or control antibodies

against cadherin 5 or carcinoembryonic antigen (CEA). After three days of incubation, DC

and T lymphocytes that appeared in the culture medium were collected. Each experiment

was conducted with 3-4 replicates per parameter tested. The number of emigrated cells

recovered from individual control explants was typically 5 x 10^5. To compare data from

different experiments, the mean number of emigrated cells in the absence of added

5   monoclonal antibody in each experiment was set equal to 1.0 and relative values were

obtained for the remaining data. Cumulative results from three independent experiments are

shown in **Figure 8**. These experiments show that monoclonal antibody to tissue factor

reduces the emigration of cells from skin explants by about 50%.

EXAMPLE 7:   **Effect of P-glycoprotein Antagonists on Dendritic Cell Migration**

10   **in Vivo**

To test the effectiveness of p-glycoprotein antagonists on dendritic cell migration in vivo,

experiments were carried out in mice. First, the murine molecule analogous to p-

glycoprotein in mouse DCs was identified. To do this, mouse skin explants, derived from

the dorsal aspect of the ear, were cultured in a manner similar to the human skin explants

15   (50). Dendritic cells (DCs) that migrated out of the lymphatic vessels of these explants

were used in efflux transport assays and in flow cytometry. Efflux of the fluorescent

substrate Fluo-3 for p-glycoprotein-related molecules was observed (**Fig. 9A**, left panel),

and flow cytometric evaluation using specific antibodies demonstrated the expression of

murine multidrug resistance related protein (MRP) (**Fig. 9B**). That MRP was the major p-

20   glycoprotein-related transporter was further evident from the observation that MRP knock-

out mice (51) failed to efflux the fluorescent substrate Fluo-3 (**Fig. 9A**, right panel).

The pharmacologic compound MK 571 is a substrate that antagonizes p-glycoprotein and MRP, but is a particularly potent inhibitor of the latter (52). Thus, MK 571 was added to cultures of human skin explants and mouse ear explants. Migration of DC was blocked by 58% and 62%, respectively (**Figs. 10 A,B**).

5   MRP knock-out mice and age-matched wild-type mice were employed in a contact sensitivity assay to determine whether MRP mediated the migration of murine DCs in vivo. Dendritic cells were analyzed for their transport of the contact sensitizer FITC to lymph nodes 24 h after its application to the skin of mice, using the contact sensitivity assay previously described (53). Dendritic cell accumulation in the lymph nodes of sensitized

10   mice was reduced by 87% in the absence of MRP expression (**Fig. 11A**).

Next, using wild-type mice, MK 571 was administered i.v. (50 mg / kg body weight) four hours prior to application of the contact sensitizer FITC to determine whether pharmacologic inhibitors of MRP and p-glycoprotein are effective in suppressing DC migration. Indeed, administration of MK 571 reduced accumulation of FITC-bearing DCs

15   in the draining lymph node by 64% (**Fig. 11B**). These data indicate that pharmacologic antagonism of p-glycoprotein suppresses dendritic cell migration.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the

invention being indicated by the appended Claims, and all changes which come within the

meaning and range of equivalency are intended to be embraced therein.

Various publications are cited herein, the disclosures of which are incorporated by reference

in their entireties.

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WHAT IS CLAIMED IS:

1.   1.   A method for decreasing the migration of dendritic cells from the peripheral tissues to

      2   the lymphatic vessels by contacting said dendritic cells with an effective amount of an

      3   agent capable of inhibiting the activity of a dendritic cell membrane protein selected

      4   from the group consisting of p-glycoprotein, tissue factor and the combination

      5   thereof.

1.   2.   The method of claim 2 wherein said agent is an antibody which binds to p-

      2   glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which

      3   blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an

      4   antisense oligonucleotide to p-glycoprotein.

1.   3.   The method of claim 3 wherein said antibodies or antibody fragments which bind to

      2   p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and

      3   antibody fragments thereof which bind p-glycoprotein.

1.   4.   The method of claim 1 wherein said agent is capable of reversing multidrug

      2   resistance.

1.   5.   The method of claim 1 wherein said agent is a calcium channel blocker, reserpine,

      2   quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,

      3   yohimbine, phenothiazines, tamoxifen, N-desmethylramoxifen, 4-hydroxytamoxifen,

      4   toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5   gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,

6   cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,

7   rapamycin, MK 571, or SR33557.

1   6.   The method of claim 5 wherein said calcium channel blocker is selected from the

2   group consisting of verapamil, diltiazem, azidopine, dihydroperidine, dexverapamil,

3   nifedipine, bepridil, nicardipine, dexniguldipine, and combinations thereof.

1   7.   The method of claim 5 wherein said cyclosporin is cyclosporine A, staurosporine,

2   PSC 833, and combinations thereof.

1   8.   The method of claim 5 wherein said phenothiazine is selected from the group

2   consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1   9.   The method of claim 1 wherein said agent is an antagonist of tissue factor.

1   10.   The method of claim 9 wherein said agent is an antibody or an antibody fragment to

2   tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a

3   recombinant fragment of tissue factor.

1   11.   The method of claim 9 wherein said agent is selected from the group consisting of

2   dilazep and retinoic acid.

1   12.   A method for suppressing the development of immunity or an immune response in a

2   mammal by contacting dendritic cells of said mammal with a therapeutically effective

3   amount of an agent capable of inhibiting the activity of a dendritic cell membrane

4   protein selected from the group consisting of p-glycoprotein, tissue factor and the

5   combination thereof.

1   13.   The method of claim 12 wherein said agent is an antibody which binds to p-

2   glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which

3   blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an

4   antisense oligonucleotide to p-glycoprotein.

1   14.   The method of claim 13 wherein said antibodies or antibody fragments which bind to

2   p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and

3   antibody fragments thereof which bind p-glycoprotein.

1   15.   The method of claim 12 wherein said agent is capable of reversing multidrug

2   resistance.

1   16.   The method of claim 12 wherein said agent is a calcium channel blocker, reserpine,

2   quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,

3   yohimbine, phenothiazines, tamoxifen, N-desmethylramoxifen, 4-hydroxytamoxifen,

4   toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5   gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,

6   cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,

7   rapamycin, MK 571, or SR33557.

1   17.   The method of claim 16 wherein said calcium channel blocker is selected from the

2   group consisting of verapamil, diltiazem, azidopine, dihydroperidine, dexverapamil,

3   nifedipine, bepridil, nicardipine, dexniguldipine, and combinations thereof.

1   18.   The method of claim 16 wherein said cyclosporin is cyclosporine A, staurosporine,

2   PSC 833, and combinations thereof.

1   19.   The method of claim 16 wherein said phenothiazine is selected from the group

2   consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1   20.   The method of claim 12 wherein said agent is an antagonist of tissue factor.

1   21.   The method of claim 20 wherein said agent is an antibody or an antibody fragment to

2   tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a

3   recombinant fragment of tissue factor.

1   22.   The method of claim 20 wherein said agent is selected from the group consisting of

2   dilazep and retinoic acid.

1   23.   The method of claim 12 wherein said suppression of immunity or the immune

2   response inhibits the immune response to an allergen.

1   24.   The method of claim 12 wherein said suppression of immunity or the immune

2   response is used for the treatment of organ transplant rejection, graft-vs.-host disease,

3   autoimmune diseases, atopic diseases, or HIV infection.

1   25.   The method of claim 24 wherein said autoimmune diseases is rheumatoid arthritis or

2   psoriasis.

1   26.   The method of claim 24 wherein said atopic disease is contact dermatitis, food

2   allergy, allergic rhinitis, allergic conjunctivitis, asthma, or eczema.

1   27.   The method of claim 12 wherein said agent is administered by the topical, parenteral,

2   ophthalmic, nasal, rectal, bronchiolar or pulmonary route.

1   28.   The method of claim 27 wherein said parenteral administration intravenous,

2   intraarterial, intramuscular, subcutaneous, or intradermal.

1   29.   A method for increasing the migration of dendritic cells from the peripheral tissues to

2   the lymphatic vessels by contacting said dendritic cells with an effective amount of an

3   agent capable of increasing the activity of a dendritic cell membrane protein selected

4   from the group consisting of p-glycoprotein, tissue factor, and the combination

5   thereof.

1   30.   The method of claim 29 wherein said agent is a p-glycoprotein agonist or a tissue

2   factor agonist.

1   31.   The method of claim 30 wherein said agent is bromocriptine, N-acetyl-leucine$_n$-

2   tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl-γ,γ'-

3   dipyridylium dichloride.

1   32.   The method of claim 29 wherein said agent is co-administered with an immunogen to

2   enhance the development of immunity to said immunogen.

1   33.   The method of claim 32 wherein said immunogen is a vaccine.

1   34.   A method for enhancing the development of immunity or an immune response in a

2   mammal by contacting dendritic cells of said mammal with a therapeutically effective

3   amount of an agent capable of increasing the activity of a dendritic cell membrane

4   protein selected from the group consisting of p-glycoprotein, tissue factor, and the

5   combination thereof.

1   35.   The method of claim 34 wherein said agent is a p-glycoprotein agonist or a tissue

2   factor agonist.

1   36.   The method of claim 34 wherein said agent is bromocriptine, N-acetyl-leucine$_n$-

2   tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl-γ,γ'-

3   dipyridylium dichloride.

1   37.   The method of claim 34 wherein said agent is co-administered with an immunogen to

2   enhance the development of immunity to said immunogen.

1   38.   The method of claim 37 wherein said immunogen is a vaccine.

2   39.   The method of claim 38 wherein said vaccine comprises a viral, bacterial, or parasite

3   immunogen.

1   40.   The method of claim 37 wherein said immunogen is a tumor antigen.

1   41.   A method for the identification of agents useful for the modulation of dendritic cell

2   migration comprising the steps of:

3   i)   identifying an agent capable of interacting with the a dendritic cell

4   membrane protein selected from the group consisting of p-glycoprotein

5   and tissue factor; and

6   ii)   determining the extent of modulation of said agent of step (i) in the

7   migration of dendritic cells in vitro or in vivo.

1   42.   A method for the identification of agents useful for modulating the development of

2   immunity or an immune response in accordance with the method of claim 41.

1   43.   A method for identifying compounds useful for modulating dendritic cell p-

2   glycoprotein activity comprising the steps of:

3   i)   loading isolated dendritic cells with a detectable substrate of the p-

4   glycoprotein transporter activity;

5   ii)   exposing said loaded cells to an agent suspected of modulating p-

6   glycoprotein activity;

7   iii)   measuring the rate of transport of said detectable substrate from said

8   dendritic cells; and

9   iv)   comparing said rate to the rate of p-glycoprotein transporter activity

10   determined of control dendritic cells not exposed to said agent,

11   wherein an increase or decreased in said rate in the presence of the agent indicates the

12   extent of activity of said agent in increasing or decreasing, respectively, p-

13   glycoprotein activity of said dendritic cells.

1   44.   A method for identifying compounds useful for modulating the migration of dendritic

2   cells by carrying out the method of claim 43, followed by determining the extent of

3   modulation of said agent of the migration of dendritic cells in vitro or in vivo.

1   45.   A method for augmenting the migration of monocytes and monocyte-derived cells

2   from tissues by contacting said monocytes with an effective amount of an agent

3   capable of augmenting monocyte p-glycoprotein activity.

1   46.   A method of treating a chronic inflammatory disease or condition in a mammal by

2   enhancing the migration of monocytes and monocyte-derived cells from the affected

3   site of said disease or condition by administration of a therapeutically effective

4   amount of an agent capable of augmenting monocyte p-glycoprotein activity.

1   47.   The method of claim 46 wherein said inflammatory disease or condition is

2   atherosclerosis, rheumatoid arthritis, or granulomatous diseases.

Figure 1

Figure 2
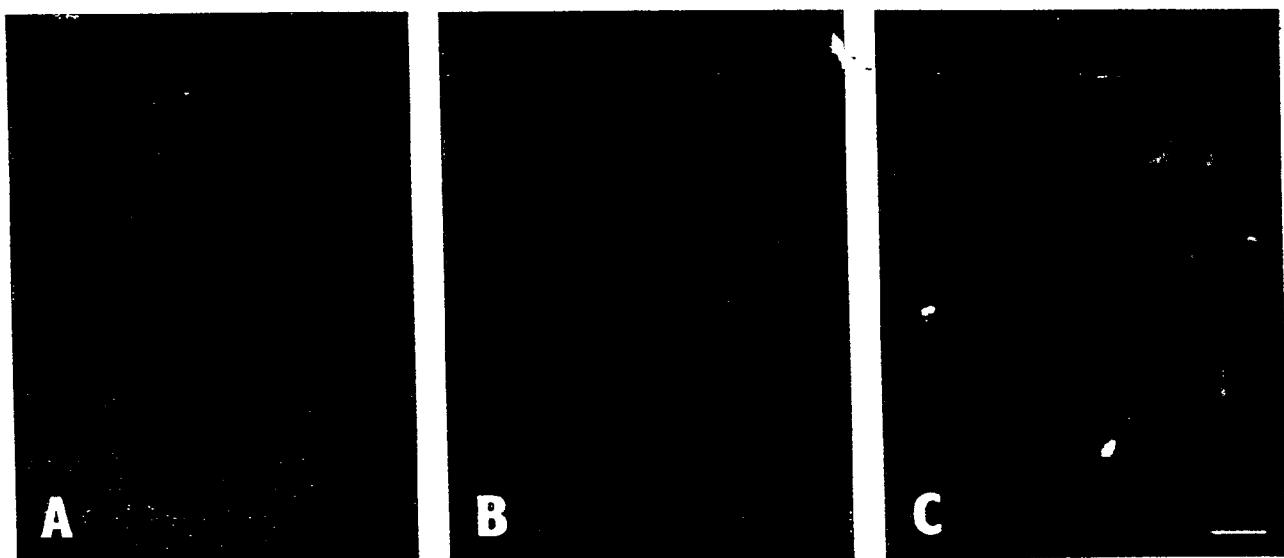
Figure 3

Figure 4

Figure 5

A
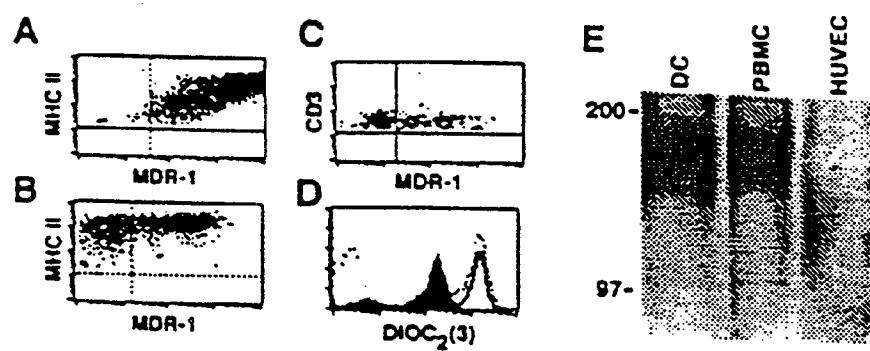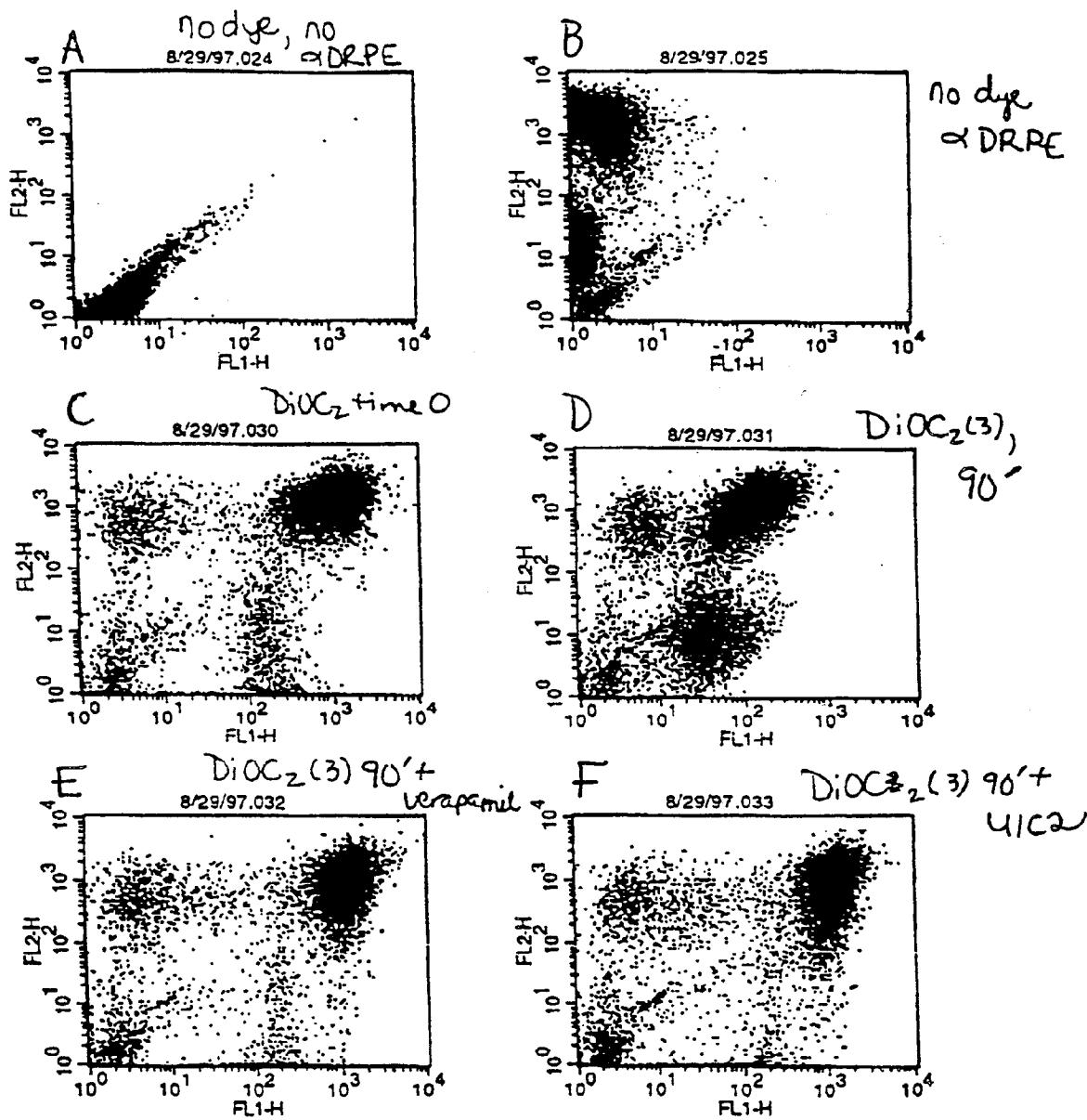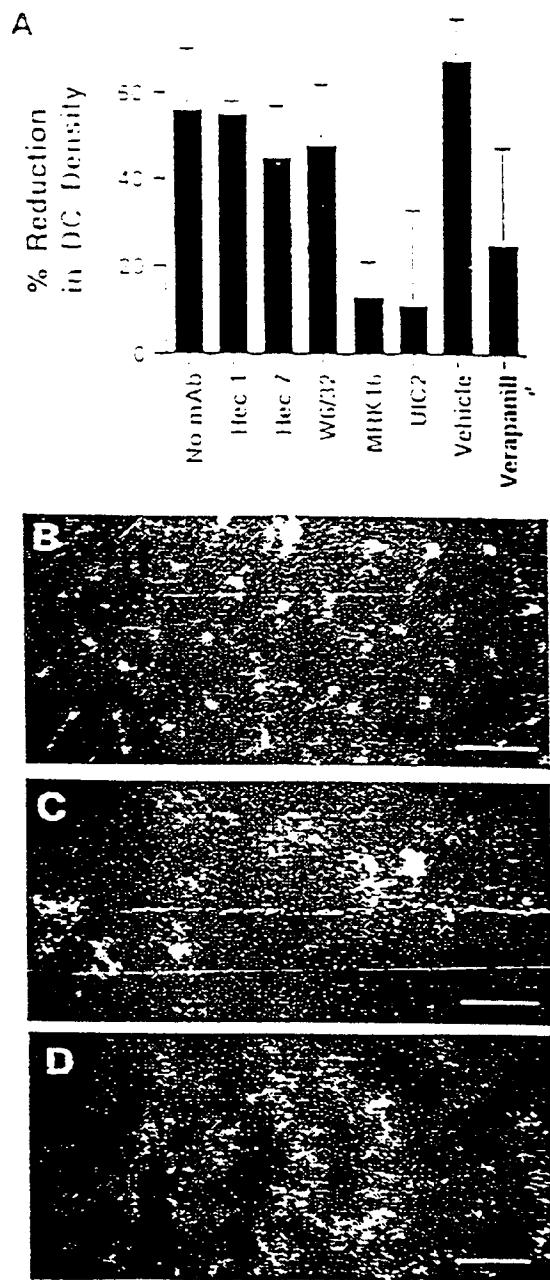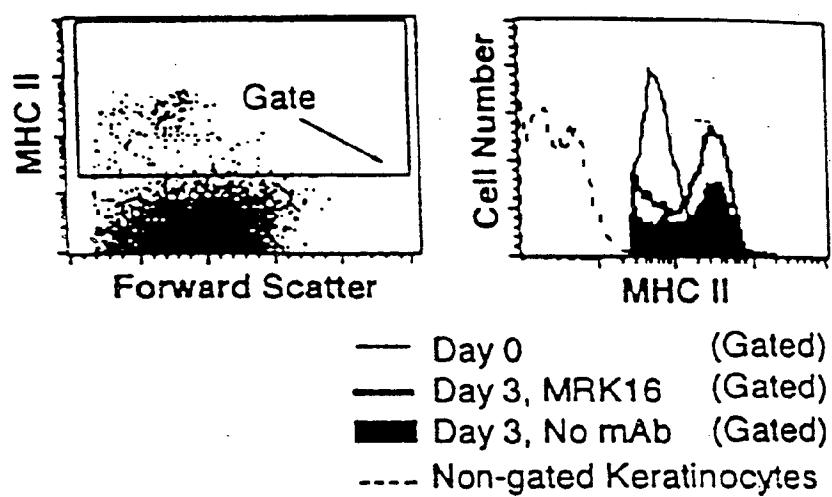
Figure 6

Figure 7

Figure 8

Figure 9

A.   **HUMAN**

B.   **MOUSE**
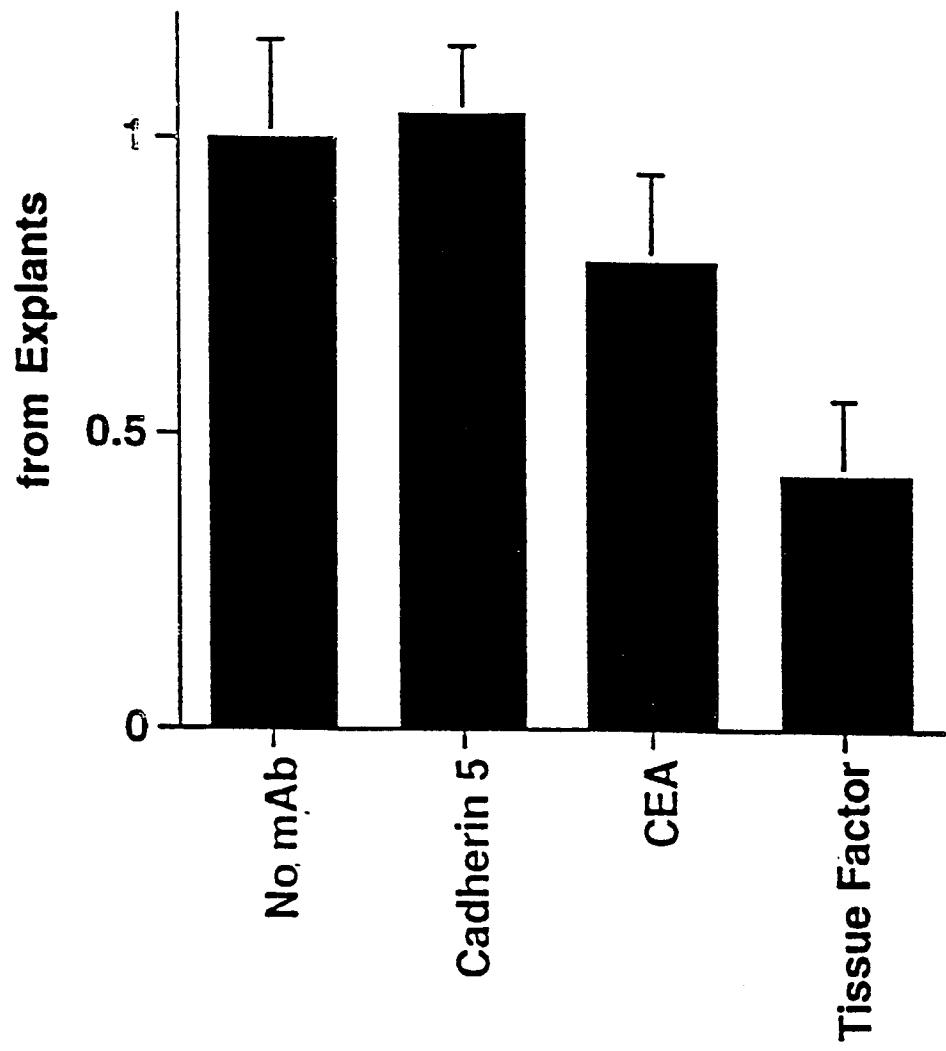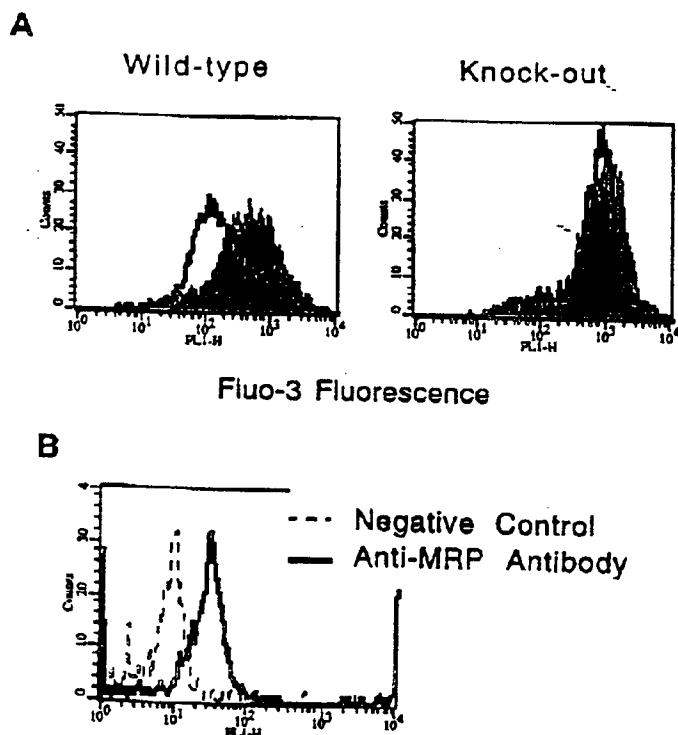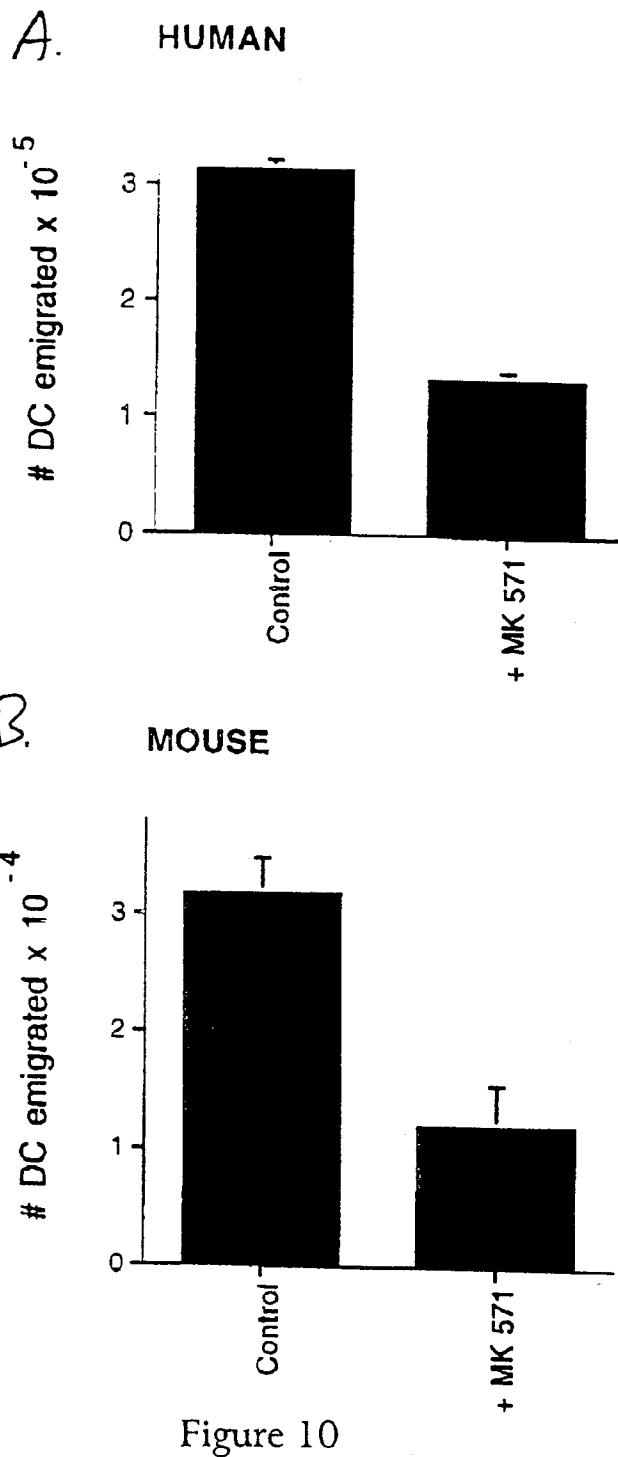
Figure 10

Figure 11

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   A61K38/00   A61K39/395   C12N15/11   G01N33/53   A61K39/39
        A61K38/13   A61K38/36   A61K31/20   A61K31/275 C07D279/00
        A61K31/55   A61K39/00   A61K39/002 A61K39/12   A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   A61K   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RANDOLPH G J ET AL: "Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro." BLOOD, (1998 DEC 1) 92 (11) 4167-77. , XP002112753 abstract page 4167, left-hand column, paragraph 3 - right-hand column, paragraph 1 page 4169, right-hand column, paragraph 2 - page 4170, right-hand column, paragraph 3 page 4172, right-hand column, paragraph 2 - left-hand column, paragraph 4 page 4175, left-hand column, paragraph 1 - right-hand column, paragraph 1 page 4176, left-hand column, paragraph 1 ---	1,9,10, 12,20, 21,23

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1999	13/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

Form PCT/ISA/210 (second sheet) (July 1992)

page 1 of 3

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   //C07K14/705,C07K14/745,C07K16/36,C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 12633 A (IMMUNEX CORP) 10 April 1997 (1997-04-10) page 2, line 31 – page 3, line 8 page 9, line 29-34 claims 16-19	29-40, 44,45
A	CUMBERBATCH M ET AL: "Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization." IMMUNOLOGY, (1995 JAN) 84 (1) 31-5. , XP002112754 page 31, left-hand column, paragraph 1 – page 32, left-hand column, paragraph 1 page 32, left-hand column, paragraph 5 page 34, left-hand column, paragraph 1 – right-hand column, paragraph 2	1-27, 41-44, 46,47

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| X | Further documents are listed in the continuation of box C. | | X | Patent family members are listed in annex. |

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1999	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 01645 A (PEPTECH UK LTD ;LEDGER PHILIP (GB)) 25 January 1996 (1996-01-25) page 1, line 6-22 page 22, line 23 - page 23, line 22 example 4 ---	1-27, 41-44, 46,47
A	MA J ET AL: "In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes." CELLULAR IMMUNOLOGY, (1994 OCT 15) 158 (2) 389-99. , XP002112755 abstract page 392, paragraph 6 - page 394, paragraph 1 page 398, paragraph 3 ---	1-47
A	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US LARSEN C P ET AL: "Migration and maturation of Langerhans cells in skin transplants and explants." retrieved from STN Database accession no. 91037811 XP002112758 abstract & JOURNAL OF EXPERIMENTAL MEDICINE, (1990 NOV 1) 172 (5) 1483-93. , ---	1-47
A	LAVIE Y. ET AL.: "Agents that reverse multidrug resistance, Tamoxifen, Verapamil, and Cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells" J. BIOL.CHEM., vol. 272, no. 3, 17 January 1997 (1997-01-17), pages 1682-87, XP002112756 abstract page 1686, paragraph 1 page 1687, paragraphs 2,3 ---	1,4-8, 12,15-19
P,X	RANDOLPH G J ET AL: "A physiologic function for p - glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUN 9) 95 (12) 6924-9. , XP002112757 the whole document -----	1-47

2

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

1-40 and 45-47 (completely), 41-44 (partially)

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1998)

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.1

Although claims 41-44 (partially) are directed to a diagnostic method
practised on the human/animal body and claims 1-40, 45-47 (completely)
are directed to a method of treatment of the human/animal body, the
search has been carried out and based on the alleged effects of the
compound/composition.

Continuation of Box I.1

Claims Nos.: 1-40 45-47 (completely), 41-44 (partially)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by
therapy
Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal
body

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter͏nal Application No

PCT/US 99/12681

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
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			AU	7392296 A	28-04-1997
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PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL MEMBRANE PROTEINS

(57) Abstract

Methods and agents are provided to decrease or increase the migration of dendritic cells for the suppression or enhancement, respectively, of the development of immunity and the immune response, by modulating the dendritic cell membrane proteins p–glycoprotein (MDR–1) and tissue factor. Agents which suppress migration have utility in the treatment of immunologically–mediated and inflammatory diseases such as graft rejection, contact dermatitis, seasonal allergies, asthma, and food allergies. Agents which enhance migration are useful for increasing the effectiveness of vaccines. Agents are also disclosed which enhance the migration of monocytes, useful in the treatment of chronic inflammatory diseases. Methods are also provided for identifying useful agents by measuring the effect on dendritic cell migration of agents which modulate p–glycoprotein and tissue factor activity, as well as the effect of agents on monocyte migration.

METHODS AND AGENTS FOR MODULATING THE IMMUNE RESPONSE AND

INFLAMMATION INVOLVING MONOCYTE AND DENDRITIC CELL

MEMBRANE PROTEINS

RESEARCH SUPPORT

FIELD OF THE INVENTION

10   The present invention relates generally to methods and agents for enhancing or suppressing

the development of immunity and the immune response by modulating the migration of

dendritic cells, and methods for identifying agents with migration modulating activity.

Methods and agents for the enhancement of monocyte migration out of chronic inflammatory

foci are also provided.

BACKGROUND OF THE INVENTION

15   The development of immunity to a specific antigen and the subsequent immune response on

re-encountering that antigen involve multi-step interactions between several immune cell

types and co-stimulatory molecules with critical spatial and temporal components. In brief,

an antigen encountered by antigen-presenting cells is taken up, processed, and its peptides

20   subsequently prime T lymphocytes to generate antigen-specific T lymphocytes (e.g.,

cytotoxic and memory cells), and through the interaction with B cells, result in the formation

of antigen-specific antibodies. Immune responses are initiated after antigen-presenting cells,

particularly dendritic cells (DC), capture antigens in organs such as skin, lung and gut, and

migrate via afferent lymphatic vessels to draining lymph nodes. T lymphocytes continually

5   recirculate through lymph nodes, so the newly arrived, antigen-bearing DC become

positioned to select lymphocytes that bear receptors for the presented antigens (1). For

example, when skin is transplanted (2), or a contact allergen such as poison ivy is applied (3),

epidermal DC (also termed Langerhans cells) migrate from their epithelial locations and carry

antigens or allergens to the lymph node. If the lymphatic conduits to the draining lymph

10   nodes are severed, the immune system does not become primed to the antigen (2). As they

migrate, the DC undergo maturation, increasing their expression of molecules involved in

antigen presentation, including major histocompatibility complex (MHC) II products, CD80

(B7-1), and CD86 (B7-2) (4, 5).

Thus, dendritic cell migration is a critical step in both the induction of immunity and in the

15   immune response. Dendritic cell migration is essential for the successful induction of an

immune response to an administered vaccine, for eliciting antibody production and cell-

mediated immunity against infectious microorganisms and arising neoplastic cells within the

body. Likewise, dendritic cell migration is responsible for diseases involving an exaggerated,

inappropriate or undesired immune response such as allergic asthma, food allergies, contact

20   dermatitis, seasonal allergies, and autoimmune diseases such as psoriasis, in which known or

as-yet undescribed antigens are transported to T lymphocytes.

White blood cells known as monocytes are precursors of dendritic cells and tissue

macrophages. These cells migrate throughout the tissues, and may develop into dendritic

cells, as described above, or macrophages, which remain in the tissues. Excessive retention

and pro-inflammatory activity of macrophages in certain tissues is implicated in acute and

5   chronic inflammatory diseases, including atherosclerosis, rheumatoid arthritis, and

granulomatous diseases.

Blockage of or interference with dendritic cell migration interferes with the immune

response. Blockage has been achieved by the use of antibodies against certain dendritic cell

proteins. Before leaving the epidermis, dendritic cells down-regulate E-cadherin, allowing

10   retraction from neighboring keratinocytes (31). In the presence of anti-α_6 integrin antibody,

epidermal DC up-regulate MHC II and retract from neighboring keratinocytes, and fail to

migrate out of the epidermis (32). Another adhesion molecule, CD44, is also used by DC

during mobilization from the epidermis (33).

It is towards improved means for specifically modulating the migration of dendritic cells

15   from the periphery to lymphatic vessels for both the positive and negative control of the

immune response, and blockage of monocyte migration to control the inflammatory response,

that the present invention is directed.

The citation of any reference herein should not be deemed as an admission that such

reference is available as prior art to the instant invention.

20   SUMMARY OF THE INVENTION

The inventors herein have made the surprising and unanticipated finding that certain dendritic

cell membrane proteins, namely p-glycoprotein, also known as MDR-1, the transporter

responsible for multi-drug resistance, and tissue factor (TF), a procoagulant protein, are

involved in the migration of dendritic cells (DCs) from the peripheral tissues to the lymphatic

5   vessels. Alteration of dendritic cell migration activity by manipulating these surface proteins

is useful for both enhancing and suppressing the immune response. Furthermore, the

migration of monocyte-derived cells out of tissues has been found to be inhibited by agents

which interfere with monocyte p-glycoprotein.

Thus, in accordance with the present invention, methods for the modulation of immunity and

10   the immune response in a mammal is provided by an agent which is capable of interacting

with the dendritic cell membrane proteins p-glycoprotein and tissue factor, and altering the

migration of dendritic cells from the peripheral tissues to the lymphatic vessels. In one

embodiment, modulation results in decreasing the migration of dendritic cells and hence the

suppression of immunity or of an immune response. Agents useful for the practice of this

15   aspect of the invention include antibodies and antibody fragments which bind to p-

glycoprotein, agents which block transport activity of p-glycoprotein, antisense

oligonucleotides to p-glycoprotein and antagonists of p-glycoprotein. Preferred agents are

those which reverse multi-drug resistance, such as calcium channel blockers, reserpine,

certain antimalarial drugs, cyclosporins, phenothiazines, tamoxifen and its metabolites,

20   certain antibiotics, antisense oligonucleotides, and other compounds known to antagonize the

activity of p-glycoprotein. Useful antibodies include MRK-16, UIC2, 4E3, and their

fragments.

In a further embodiment, antagonists of tissue factor are useful for the suppression of the development of immunity and of the immune response. Such antagonists include antibodies and antibody fragments to tissue factor, tissue factor antisense oligonucleotides, fragments of tissue factor, and compounds known to antagonize tissue factor such as dilazep and retinoic

5   acid.

Suppression of the immune response is useful for the treatment of several diseases and conditions. For example, allergic reactions, graft-vs-host disease, transplant rejection, autoimmune diseases, progression of HIV infection, contact dermatitis, and food allergy are treatable by the methods and agents of the present invention. The agents may be

10   administered by appropriate routes including topical, parenteral, ophthalmic, nasal, pulmonary, etc., for delivery to the site of encounter of the dendritic cells with the antigen and to prevent their subsequent migration to the lymphatic vasculature.

In a further embodiment, certain methods and agents of the present invention may be used which enhance the migration of dendritic cells from the peripheral tissues to the lymphatic

15   vessels. These methods and agents promote the development of immunity or of an immune response to a newly encountered antigen and enhance immunity or the immune response on re-encountering an antigen. Agents useful in the practice of this aspect of the present invention are agonists of the dendritic cell membrane proteins p-glycoprotein and tissue factor. Examples of useful agents include bromocriptine, N-acetyl-leucine-leucine-tyrosine

20   amide, and cytarabine for p-glycoprotein. In a preferred embodiment, the agent is co-administered with an immunogen to enhance the development of an immune response to said immunogen, for example, when the immunogen is a vaccine.

In a further aspect of the present invention, methods for the identification of agents useful for the modulation of dendritic cell migration is provided. These methods comprise the steps of first identifying an agent capable of interacting with the dendritic cell membrane proteins p-glycoprotein and tissue factor, followed by determining the extent of modulation of the

5   migration of dendritic cells in vitro or in vivo by the agent. These methods identify agents useful for modulating the development of immunity or an immune response.

In another aspect of the present invention, methods for the modulation of inflammation in a mammal are provided by an agent which is capable of enhancing the function of the monocyte membrane protein p-glycoprotein, and increasing the migration of inflammatory

10   monocytic cells from the tissues. Agents useful for the practice of this aspect of the invention include the aforementioned agonists of p-glycoprotein. Increasing monocyte migration out of tissues is useful in the treatment of such inflammatory conditions as atherosclerosis, rheumatoid arthritis and granulomatous diseases.

In a still further aspect of the present invention, methods for the identification of agents

15   useful for the augmentation of monocyte migration out of tissues is provided. These methods comprise the steps of first identifying an agent capable of enhancing the monocyte membrane protein p-glycoprotein, followed by determining the extent of augmentation of the migration of monocytes in vitro or in vivo by the agent. These methods identify agents useful for treating inflammatory conditions and diseases.

20   These and other aspects of the present invention will be better appreciated by reference to the following figures and Detailed Description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 A-B. Effect of anti-MDR-1 monoclonal antibodies (mAbs) on transendothelial

migration of mononuclear phagocytes. (A) Monocytes were allowed to migrate across

endothelial monolayers with or without addition of anti-MDR-1 mAb MRK16, or mAb to b_2

5   integrin (IB4) or CD31 (hec 7). Cultures were incubated for 1.5 hr. (B) To examine

subsequent basal-to-apical transmigration, monocytes were first allowed to accumulate

beneath the endothelium in the absence of added mAb for 1.5 hours, followed by addition of

mAbs (all IgG2a; MRK16 used as F(ab')$_2$ fragments) at the indicated concentrations; in some

samples, monocytes were preincubated with MRK16 or isotype-matched control mAb hec 1

10   against cadherin 5 (Pre-MRK16 and Pre-Hec 1) before addition to the endothelium. After 48

hr, cultures were fixed for analysis. Data are plotted relative to control levels of reverse

transmigration (no mAb added), in which 50% of the subendothelial MP retraverse the

endothelium in 48 hr. Bars represent means $\pm$ SD from three to ten experiments, except

UIC2 which was tested in a single experiment.

15   **Figure 2.** Effect of anti-MDR-1 mAb on emigration of DC and T lymphocytes from skin

explants. Explants of human skin were floated in culture medium without added mAb (n =

10) or in medium containing anti-MDR-1 mAbs MRK16 (n = 10) or UIC2 (n = 3),

anti-cadherin 5 mAb hec 1 (n = 5), anti-CD31 mAb hec 7 (n =3), anti-MHC I mAb W6/32 (n

= 3), anti-CD18 mAb IB4 (n = 2), verapamil (n =3), or the vehicle control for verapamil,

20   0.03% methanol (Vehicle, n = 1). After three days of incubation, DC and T lymphocytes that

appeared in the culture medium were collected and counted as previously described (7). Each

experiment was conducted in triplicate; n = number of experiments in which condition was

included. The number of emigrated cells recovered from individual control explants was typically 5×10^5. To compare data from different experiments, the mean number of emigrated cells in the absence of added mAb in each experiment was set equal to 1.0 and relative values were obtained for the remaining data.

5 **Figure 3 A-C**. Expression of MDR-1 in situ by DC. (**A**) Epidermis derived from explants cultured with anti-MDR-1 mAb MRK16 were fixed and Cy3-conjugated anti-mouse IgG was added to detect expression of MDR-1 (red). Addition of Cy3-labeled detection antibody to skin incubated in the presence of nonbinding control mAb (Hec1) showed no staining (not shown). (**B**) Immunostaining of same sample using FITC-conjugated anti-MHC II mAb

10 (green) identified dendritic cells. (**C**) Doubly-exposed frame to examine co-localization of p-glycoprotein and MHC II on dendritic cells (yellow). Bar is 10 μm.

Figure 4 A-E. Expression of functional MDR-1 by emigrated DC. (**A-C**) MDR-1 expression in emigrated skin cells was examined by flow cytometry using double-staining with anti-MDR-1 mAb and the DC lineage marker MHC II (**A, B**) or the T lymphocyte lineage

15 marker CD3 (**C**). Quadrants were marked based on the level of fluorescence intensity observed in samples stained with negative control mAbs. (**D**) For studies measuring efflux, emigrated cells were loaded with the dye 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$) (- - -, no dye added) and cultured at 37 °C for 90 minutes without addition of mAb (filled profile), in the presence of UIC2 mAb (bold line), or kept at 4 °C for this duration (thin line).

20 Analysis of efflux in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-MHC II mAb for flow cytometry. Figure shown was gated on MHC II$^+$ cells. (**E**) Membranes prepared from purified, emigrated DC, PBMC, or HUVEC were

immunoblotted with anti-MDR-1 mAb C219. Numbers to the left of bands are molecular

weight markers.

Figure 5 A-F. Effect of verapamil on the efflux of the p-glycoprotein synthetic substrate

3,3'-diethyloxacarbocyanine iodide (DiOC$_2$) from dendritic cells. Panels A and B show the

5   fluorescence intensity of dendritic cells before loading with DiOC$_2$. In these cells, the FL1

staining intensity (x-axis) is low. Panels C-F show dendritic cells incubated under various

conditions after loading with DiOC$_2$, which fluoresces on the FL1 channel. Panels B-F show

cells after labeling for the cell surface marker MHC II (FL2 channel). Cell that shift 10^3

staining intensity on the FL2 channel are dendritic cells. For efflux measurements, dendritic

10   cells emigrated from skin were loaded with the dye and analyzed immediately for dye content

(time 0, Panel C), or cultured for 90 minutes at 37°C without additives (D) or in the presence

of verapamil (10 μg/ml) (Panel E) or the anti-p-glycoprotein mAb UIC2 (10 μg/ml) (Panel

F).

Figure 6 A-D. Retention of DC in the epidermis after treatment with MDR-1 antagonists.

15   (A) Epidermal sheets were stained with anti-MHC II mAb to enumerate DC before the onset

of culture or after three days of culture in the absence of mAb (no mAb, n = 7), or in the

presence of anti-cadherin 5 mAb hec 1 (n = 3); anti-MHC I mAb W6/32 (n = 3); anti-CD31

mAb hec 7 (n = 3); anti-MDR-1 mAb MRK16 (n = 6); anti-MDR-1 mAb UIC2 (n = 1);

verapamil (n = 2); or the vehicle control for verapamil, 0.03% methanol (Vehicle, n = 1). n =

20   number of experiments in which each condition was examined. DC were counted from *en*

face examinations of epidermal sheets in 16 to 20 high-power fields per experiment. Percent

reduction in DC density was calculated by comparing the number of DC in cultured explants

to the mean number present in a portion of the same skin sample before culture (typically 75

cells/field). **B-D)** Photomicrographs show the distribution of DC within the epidermis before

culturing of explants **(B)**, after three days of culture under control conditions (no mAb) **(C)**,

and after three days of culture in the presence of MRK16 **(D)**. Bar is 20 µm.

5   **Figure 7**. Effect of anti-MDR-1 mAb on the maturation of DC. The levels of MHC II

expressed by epidermal DC were analyzed in gated epidermal suspensions by flow cytometry

before the onset of culture (Day 0) or after three days of culture in the absence of mAb or in

the presence of MRK16. Single cell suspensions of the epidermis were prepared by digestion

with dispase followed by trypsin. Keratinocytes and other skin cells were excluded from the

10   analysis by setting a gate to include only MHC II$^+$ cells.

Figure 8. Effect of anti-tissue factor monoclonal antibody (mAb) on emigration of dendritic

cells (DC) and T lymphocytes from skin explants. Explants of human skin were floated in

culture medium without added mAb or in medium containing anti-tissue factor mAb VIC7 or

control mAbs against cadherin 5 or carcinoembryonic antigen (CEA). After three days of

15   incubation, DC and T lymphocytes that appeared in the culture medium were collected.

Cumulative results from three independent experiments are shown. Each experiment was

conducted with 3-4 replicates per parameter tested. The number of emigrated cells recovered

from individual control explants were typically 5×10^5. To compare data from different

experiments, the mean number of emigrated cells in the absence of added mAb in each

20   experiment was set equal to 1.0 and relative values were obtained for the remaining data.

Figure 9 A-B. Analysis of expression of p-glycoprotein-related transporters in mice. A)

Dendritic cells emigrated from wild-type or MRP knock-out mouse ear explants were

loaded with the fluorescent transporter substrate Fluo-3 and cultured at 37° C for 60

minutes (open profile) or kept at 4° C for this duration (filled profile). Analysis of efflux

5   in the DC fraction of emigrants was assessed by double labeling with PE-conjugated anti-

MHC II mAb for flow cytometry. Figure shown was gated on MHC II$^+$ cells. B) MRP

expression in emigrated skin cells was examined by flow cytometry in permeabilized cells

using double-staining with anti-MRP mAb MRPr1 (Signet Laboratories) or rat control mAb

R35-95 (Pharmingen), followed by FITC-conjugated anti-rat mAb for detection.

10   **Figure 10 A-B.** Effect of MRP antagonist MK 571 on emigration of DC from skin

explants. Explants of human skin or explants of the dorsal aspect of mouse ear were floated

in culture medium with or without addition of MK 571 (25 μM). Murine emigrated

dendritic cells were collected and explants were transferred to fresh medium daily (with or

without MK 571) and were analyzed over 5 days. Human skin explants were analyzed on

15   day 2, with a medium change or fresh addition of drug at day 1. Each experiment was

conducted using 3 (human) to 10 (mouse) replicates and was performed on at least two

independent occasions.

Figure 11 A-B. Contact sensitization in MRP knock-out mice or after administration of

MRP antagonist to wild-type mice. A) Twenty five microliters of FITC dissolved in

20   acetone:dibutylphthalate (8 mg/ml stock) was applied to the dorsothoracic area of shaved

mice. Twenty four hours later, draining lymph nodes were collected, disaggregated to

obtain single cell suspensions, and stained for the dendritic cell marker CD11c. CD11c

was detected using biotinylated anti-CD11c and streptavidin-conjugated R-phycoerythrin.

Data are from a single experiment performed in quadruplicate. B). Mice were injected

with 1 mg MK 571 intraperitoneally before application of FITC and analysis as described

5   in (A). Data are from 2 experiments, each performed with triplicate samples.

DETAILED DESCRIPTION OF THE INVENTION

The following terms shall have the definitions set out herein. The terms "immunity" or

"development of immunity" are used generally to refer to the initial encounter of the immune

system with an antigen and the development of a specific immune response to the antigen.

10   This entails, in brief, the uptake of the antigen by antigen-presenting cells (APCs), especially

dendritic cells (DC), processing of the antigen, and presentation of antigen peptides in a

complex with MHC molecules, to T lymphocytes, with the subsequent formation of antigen-

specific T lymphocytes, including memory, helper, and cytotoxic T lymphocytes, as well as

antibody-producing B lymphocytes. The term immune response is generally used herein to

15   refer to the response of the immune system to a subsequent encounter with an antigen to

which immunity has been previously established. One aspect of this encounter is the uptake

of the antigen by APCs, migration to draining lymphoid organs including lymph nodes and

the spleen, and subsequent presentation to memory T lymphocytes that circulate in lymphoid

tissues.

20   The terms "inflammation" and "inflammatory response" refer to the acute or chronic response

to a known or unknown antigen in which a particular general or local site within the body

exhibits adverse sequelae of the accumulation of white blood cells, including monocytes, and

their secreted mediators, producing a range of effects of varied intensities including pain,

tenderness, edema, destruction of tissue, organ failure, asthma, and urticaria (hives). In the

case of atherosclerosis, a chronic inflammatory disorder of the arterial wall, accumulation and

retention of monocytes in the intimal tissue of the artery leads to lipid-laden plaque formation

5   and danger of thrombosis.

As described above, the present invention relates to methods and agents for the modulation of

the immune response involving the dendritic cell membrane proteins p-glycoprotein and

tissue factor. As will be seen in the examples below, these proteins have been discovered to

have an important physiological role in the migration of dendritic cells from the peripheral

10   tissues, a location where antigen is encountered, to the lymphatic vessels, where interaction

of the dendritic cell with T lymphocytes induces immunity or an immune response.

Manipulation of these proteins to antagonize or agonize their activities has been found to be

useful for decreasing or increasing, respectively, the ability of the dendritic cell to migrate to

the lymphatic vessels and thus modulate the development of immunity or an immune

15   response to an antigen or antigens encountered by dendritic cells.

p-Glycoprotein, also known as MDR-1, is a large plasma membrane protein that functions as

an ATP-driven chemotherapeutic drug efflux pump. The endogenous substrate(s) for the p-

glycoprotein transporter has not yet been identified, but based on structure-activity

relationships of numerous compounds, are believed to include cationic, hydrophobic

20   molecules with at least two planar rings and a molecular weight of 400-1500 (40).

Endogenous substrates also likely include modified phospholipids, such as phospholipids that

are involved in cell signaling. The presence of p-glycoprotein on cells confers multi-drug

resistance ("MDR"); in cancer cells, p-glycoprotein pumps out of the cell chemotherapeutic

agents and thus renders cancer cells resistant to chemotherapy (36). Thus, to enable or

improve cancer chemotherapy in resistant tumors, p-glycoprotein has been a target for

pharmacologic intervention to disable the pump (36). Numerous compounds have been

5   found which antagonize the activity of the pump, and several are in use or in clinical

development as adjunct therapy with cancer chemotherapeutic agents to block resistance.

Prophylactic use has also been suggested to prevent the appearance of a drug resistant

phenotype.

Several gene products that are highly homologous to p-glycoprotein also confer drug

10   resistance and can transport a similar profile of substrates as p-glycoprotein. Examples of

these related proteins include cMOAT (canalicular multiple organic anion transporter), LRP

(lung resistance protein), and MRP (multi-drug resistance related protein). Expression of

these p-glycoprotein-related gene products in monocytes or dendritic cells of humans or other

mammalian species may confer the same function as the MDR-1 gene product. Therefore,

15   p-glycoprotein as defined herein embraces the MDR-1 gene product or the gene product(s) of

structurally and functionally related transporters that mediate dendritic cell migration in

humans and other mammals.

Methods and agents which have been found to effectively antagonize the activity of the p-

glycoprotein protein are useful in the practice of the present invention for antagonizing p-

20   glycoprotein and thus inhibiting dendritic cell migration and suppressing immunity or an

immune response. These methods and agents comprise several approaches, including but not

limited to antibodies and fragments which bind to p-glycoprotein, compounds which reverse

multi-drug resistance, and means to reduce the expression of p-glycoprotein. These methods

and agents may be used singly or in combination. As will be described in more detail below,

administration may be targeted to a particular location of the body where the activity is

desired, for example on or in an cutaneous lesion such as psoriasis or contact dermatitis, and

5   delivery by aerosol or powder to the bronchi or lungs in the case of asthma.

Suppression or down-regulation of immunity and the immune response has utility in the

prophylaxis and treatment of numerous diseases and conditions. Methods and agents of the

present invention are useful as prophylaxis against the exposure of an individual to a known

immunogen or allergen against which an immune response would be highly undesirable. For

10   example, acute exposure to a causative agent of contact dermatitis, such as might occur in an

industrial setting, for example, exposure to protein-modifying metals and their salts such as

nickel and chromate as a result of a chemical spill, or release of a gaseous or airborne

particulate allergen; a further example is topical or pulmonary exposure to urushiol, from

contact with poison ivy or inhalation of smoke from burning foliage. Immunity to accidental

15   oral consumption of a potent immunogen may be blocked by the methods and agents of the

present invention. An individual exposed under such conditions can prevent the delivery of

the antigen to the lymph nodes by the methods and agents of the present invention.

Wider utility of the methods and agents of the present invention are found in the treatment of

acute and chronic conditions mediated by the initial or repeat exposure of the immune system

20   to a known or as-yet unidentified antigen causative of a condition or disease. A role for

dendritic cells in the development of immunity has been established; the role of dendritic

cells in the subsequent immune response has been established for many antigens and is

suspected in others. The agents of the present invention that suppress the migration of

dendritic cells are useful for one or both of these aspects, and especially in the type of

immune response known as delayed-type hypersensitivity and more particularly, contact

hypersensitivity. Examples of known immunogens that cause disease include pollen and

5   other airborne allergens which cause allergic rhinitis and other seasonal allergic reactions,

hay fever, allergic conjunctivitis; arthropod-borne antigens such as dist mites and insect

secretions such as those that cause the reaction to mosquito bites; foods that cause allergies,

etc. In numerous autoimmune diseases, suspected antigens to which an immune response is

responsible for the pathology have been identified; in others, the antigens are probable, and in

10   others, they are uncertain. Examples of as-yet identified immunogens responsible for

pathological inflammatory conditions include food allergies of unknown cause, irritable

bowel syndrome, rheumatoid arthritis, psoriasis and a large number of autoimmune diseases

which are believed to be initiated and maintained by endogenous antigens. In a further utility

of the present invention, means to block an immune response to transplanted antigens, in the

15   form of acute rejection, is provided. This may be used as an adjunct to immunosuppressive

and other agents routinely used in transplantation.

The methods and agents of the present invention are not limited to, but are particularly useful

in, the treatment of inflammatory conditions where the acute or chronic exposure to antigen

occurs in the periphery, such as the skin, as well as the gut and the lungs.

20   As mentioned above, one particular condition amendable to treatment by the methods and

agents of the present invention is in the area of transplantation. A critical role has been

identified in the interaction of antigen-presenting cells, dendritic cells in particular, with T

lymphocytes in initiating rejection. It is well established that dendritic cells mediate allograft

rejection, since experimental techniques used to deplete allografts of endogenous dendritic

cells do not give rise to subsequent rejection responses in recipients. However, it is not

clinically feasible to deplete allografts of endogenous dendritic cells. Because it is the

5   migration of dendritic cells to the graft recipient's lymph nodes and spleen that ultimately

results in graft rejection, clinically feasible methods to block dendritic cell migration to these

lymphoid tissues would prevent graft rejection. Suppression of the migration of dendritic

cells by agents which antagonize p-glycoprotein or tissue factor and thus inhibit their

interaction with T lymphocytes blocks the development of the immune response and thus are

10   useful for averting graft rejection.

Another disease particularly amenable to both prophylaxis treatment by the methods and

agents of the present invention is HIV. Transepithelial infection by HIV is believed to be

mediated by dendritic cells, which become infected with the virus and carry the virus to

lymph nodes where interactions between dendritic cells and T lymphocytes as described

15   above lead to infection of T lymphocytes. It is known that bone marrow-derived dendritic

cells facilitate the creation of a population of CD 4+ T lymphocytes, which are a major

reservoir of HIV infection. Methods and agents of the present invention which block the

migration of dendritic cells from peripheral sites, where dendritic cell HIV infection occurs,

can interrupt the endogenous spread of infection; suppressing further dendritic cell migration

20   inhibits the dendritic cell-mediated fostering of infected T lymphocyte populations.

In one embodiment of the present invention, antibodies or antibody fragments to p-

glycoprotein may be administered to inhibit dendritic cell migration. Antibodies may be

made which bind to the extracellular portion of the p-glycoprotein molecule. Non-limiting

examples of suitable antibodies include monoclonal antibodies MRK16 (24), 4E3 (23), and

UIC2 (22). Such antibodies include but are not limited to polyclonal, monoclonal, chimeric,

single chain, Fab fragments, and an Fab expression library. Other fragments of such

5   antibodies capable of binding to an epitope on the p-glycoprotein molecule are embraced

herein. The anti-p-glycoprotein antibodies of the invention may be cross reactive, *e.g.*, they

may recognize p-glycoprotein from different species. Polyclonal antibodies have greater

likelihood of cross reactivity. Alternatively, an antibody of the invention may be specific for

a single form of p-glycoprotein, such as murine p-glycoprotein. Preferably, such an antibody

10   is specific for human p-glycoprotein.

Various procedures known in the art may be used for the production of polyclonal antibodies

to p-glycoprotein. For preparation of monoclonal antibodies directed toward p-glycoprotein,

or fragment thereof, any technique that provides for the production of antibody molecules by

continuous cell lines in culture may be used. These include but are not limited to the

15   hybridoma technique originally developed by Kohler and Milstein [*Nature* **256**:495-497

(1975)], as well as the trioma technique, the human B-cell hybridoma technique [Kozbor et

al., *Immunology Today* 4:72 1983); Cote et al., *Proc. Natl. Acad. Sci. U.S.A.* **80**:2026-2030

(1983)], and the EBV-hybridoma technique to produce human monoclonal antibodies [Cole

et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 (1985)].

20   In an additional embodiment of the invention, monoclonal antibodies can be produced in

germ-free animals utilizing recent technology [PCT/US90/02545]. In fact, according to the

invention, techniques developed for the production of "chimeric antibodies" [Morrison et al.,

J. Bacteriol. **159**:870 (1984); Neuberger et al., *Nature* **312**:604-608 (1984); Takeda et al.,

Nature 314:452-454 (1985)] by splicing the genes from a mouse antibody molecule specific

for p-glycoprotein together with genes from a human antibody molecule of appropriate

biological activity can be used; such antibodies are within the scope of this invention. Such

human or humanized chimeric antibodies are preferred for use in therapy of human diseases

5   or disorders (described *infra*), since the human or humanized antibodies are much less likely

than xenogenic antibodies to induce an immune response, in particular an allergic response,

themselves.

According to the invention, techniques described for the production of single chain antibodies

[U.S. Patent Nos. 5,476,786 and 5,132,405 to Huston; U.S. Patent 4,946,778] can be adapted

10   to produce p-glycoprotein-specific single chain antibodies. An additional embodiment of the

invention utilizes the techniques described for the construction of Fab expression libraries

[Huse et al., *Science* 246:1275-1281 (1989)] to allow rapid and easy identification of

monoclonal Fab fragments with the desired specificity for p-glycoprotein.

In a second, and preferred, embodiment of the present invention, administration of agents to

15   inhibit dendritic cell migration are provided which are known to reverse multiple drug

resistance. Suitable agents may be selected from compounds that inhibit this drug

transporter, including such non-limiting examples as calcium channel blockers such as

verapamil, diltiazem, dihydroperidine, nifedipine, bepridil, and nicardipine; other

cardiovascular drugs, including amiodarone, dipyridamole, and quinidine; antimalarial

20   compounds such as quinine, quinacrine, and cinchonine; cephalosporin and other antibiotics

such as cefoperazone, ceftriaxone, erythromycin, valinomycin, bafilomycin, and gramicidin;

cyclosporins such as cyclosporine A and staurosporine; phenothiazines such as

trifluoperazine, chlorpromazine and fluphenazine; hormones such as tamoxifen,

N-desmethylramoxifen, 4-hydroxytamoxifen, toremifene, and progesterone, and other

compounds such as terfenadine, monensin, yohimbine, reserpine, cremophor, benzquinamide,

estramustine, and tacrolimus.

5   In a preferred embodiment, more potent inhibitors of multi-drug resistance than those

described above are used in the practice of the present invention. Non-limiting examples

include the cyclosporine analog PSC 833, MK 571, and the calcium channel blockers

dexverapamil and dexniguldipine. PSC 833 is a cyclosporine analog with greater binding

specificity for p-glycoprotein (36); it is 10-fold more potent than cyclosporine A and can

10   reverse multi-drug resistance in vitro at concentrations of 0.5 to 2.0 μmol/L. MK 571 is a

substrate that antagonizes p-glycoprotein and murine multidrug resistance related protein

(MRP), and is a particularly potent inhibitor of the latter. MK 571 is known to be an

antagonist of the leukotriene D_4 receptor (54), and as will be seen in the Examples below, has

utility for the purposes described herein. Other potent inhibitors of p-glycoprotein include

15   S9788, rapamycin, MK 571, GF120918, and SR33557.

In a further embodiment, inhibition of p-glycoprotein in dendritic cells may be achieved by

targeting gene expression with antisense oligonucleotides. This approach has been

demonstrated feasible in vitro using mouse 3T3 fibroblasts (41).

As described above, another dendritic cell surface protein that may be antagonized to achieve

20   the practice of the present invention is tissue factor (abbreviated TF). Tissue factor is a

procoagulant protein present on the surface of numerous cell types, including endothelial

cells, which line the vasculature, and monocytes, which circulate in blood. Increased

expression of tissue factor by these cell types has been linked to many thrombotic disorders

and pathologic states (see below with regard to increased expression). The majority of

monocyte membrane-associated tissue factor is not in an enzymatically active form that can

5   initiate clotting; in order to become active, tissue factor must form an active complex with

another of the clotting factors, Factor VII or its activated form, Factor VIIa. This complex

then leads to clot formation.

Also as described above in the instance of antagonists of p-glycoprotein, tissue factor may

also be antagonized in order to inhibit dendritic cell mobility. This may be achieved by the

10   use of antibodies and fragments against tissue factor. A monoclonal antibody against tissue

factor that inhibits migration of DC is VIC7. Compounds known to inhibit tissue factor

activity include the dilazep (42) and retinoic acid (43). In a further embodiment, inhibition of

tissue factor expression in dendritic cells may be achieved by targeting gene expression with

antisense oligonucleotides to tissue factor (49). A further strategy on interfering with tissue

15   factor activity in order to suppress dendritic cell migration is through the use of purified,

nonlipidated fragments of soluble recombinant human TF. Migration was inhibited in the

presence of a soluble, recombinant form of human TF representing the complete amino acid

sequence of the extracellular region or fragments thereof, expressed in E. coli and used in

delipidated form. Soluble TF impeded migration by $69 \pm 2\%$ in eight independent

20   experiments when used at 5-20 μg/ml. Only fragments containing amino acid residues that

are carboxyl to residue 202 blocked reverse transmigration effectively. This finding agrees

well with the location of the epitope for mAb VIC7, which is between amino acids 181-214.

The finding that both anti-TF mAb and soluble TF inhibit reverse transmigration is consistent

with a model in which the binding of endogenous TF on monocytes to an essential ligand is

prevented by anti-TF mAb and is competed with by soluble recombinant TF. The

competitive binding sites contained in the region spanning 202-219.

5   Enhancing the effectiveness of vaccination and other forms of immunotherapy is another

feature of the present invention. This aspect is carried out using methods and agents which

enhance or activate the dendritic cell membrane proteins p-glycoprotein and tissue factor.

Agents useful for this utility include activators of p-glycoprotein, such as bromocriptine (44),

N-acetyl-leucine$_n$-tyrosine amide where n=1-6 (45), and cytarabine (46) are preferred.

10   Suitable activators of tissue factor include endotoxin (47), silver ion (47), and N,N'-dimethyl-

γ,γ'-dipyridylium dichloride (48). Preferably, these agents are admixed with the immunogen

to be used for vaccination.

A further utility of one aspect of the present invention is in the immunotherapeutic treatment

of cancer. Numerous studies and trials involve attempts to initiate or enhance an immune

15   response in the patient toward the patient's own tumor. However, these regimens have

provided little positive results, often because the patient is unable to mount an effective

immune response to the tumor antigens. In this case, effective antigen presentation maybe

suppressed. The methods and agents of the present invention directed towards the

enhancement of the immune response and increasing the effectiveness of vaccines has

20   particular utility in the enhancement of therapeutic vaccines for cancer.

In another embodiment of the present invention, methods and agents may be used to increase dendritic cell migration in order to achieve an enhancement of the development of immunity or in a subsequent immune response to an antigen. This would be carried out using agents that increase transport of endogenous substrates for p-glycoprotein that mediate dendritic cell

5   migration. Enhanced development of immunity is particularly useful in enhancing the effectiveness of a vaccine, such as that for viruses such as poliomyelitis, rubella, measles, variola, and varicella, and especially poorly immunogenic immunogens such as influenza; bacteria such as *Borrelia burgdorferi*, the causative pathogen of Lyme disease, and other organisms including parasites such as malaria. Furthermore, the invention herein is also

10   useful in the various newer immunotherapeutic approaches to the prevention and control of other infectious diseases such as HIV and hepatitis, and immunotherapies of various cancers (infra). In these approaches, potentially immunogenic components of the virion or tumor are prepared by various means and introduced into the patient in an attempt to develop immune recognition of the pathologic antigen(s). Often, the patient is immunocompromised and

15   cannot mount an effective immune response to the antigen. One embodiment of the methods and agents of the present invention provide a means for increasing the delivery of antigen to the immune system by increasing or enhancing the migration of dendritic cells which have processed the antigen to the lymphatic vessels, where they may subsequently encounter T lymphocytes and induce cellular and humoral immunity.

20   The route of delivery of the agents of the present invention may be selected by one skilled in the art to provide the optimal contact with dendritic cells involved in the etiology of the condition to be treated or the route of exposure of the immunogen. As examples, contact dermatitis, psoriasis, and other topical conditions may be treated by application of the agent

of the present invention to the skin. Allergic conjunctivitis may be treated by application of

the agent to the eye. Parenteral or other routes of administration which results in generalized

delivery of the agent is also provided. Bronchiolar and pulmonary exposure may be achieved

by aerosol and fine powder inhalation, respectively.

5 In yet another aspect of the present invention, provided herein are pharmaceutical

compositions of the above agents. Such pharmaceutical compositions may be for

administration for injection, or for oral, pulmonary, nasal or other forms of administration. In

general, comprehended by the invention are pharmaceutical compositions comprising

effective amounts of a low molecular weight component or components, or derivative

10 products, of the invention together with pharmaceutically acceptable diluents, preservatives,

solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of

various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength; additives

such as detergents and solubilizing agents (*e.g.*, Tween 80, Polysorbate 80), anti-oxidants

(*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol)

15 and bulking substances (*e.g.*, lactose, mannitol); incorporation of the material into particulate

preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into

liposomes. Hylauronic acid may also be used. Such compositions may influence the physical

state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and

derivatives. *See, e.g.*, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack

20 Publishing Co., Easton, PA 18042) pages 1435-1712 which are herein incorporated by

reference. The compositions may be prepared in liquid form, or may be in dried powder,

such as lyophilized form.

Contemplated for use herein are oral solid dosage forms, which are described generally in

Remington's Pharmaceutical Sciences, 18th Ed.1990 (Mack Publishing Co. Easton PA

18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include

tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid

5   encapsulation may be used to formulate the present compositions (as, for example, proteinoid

microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used

and the liposomes may be derivatized with various polymers (e.g., U.S. Patent No.

5,013,556). A description of possible solid dosage forms for the therapeutic is given by

Marshall, K. In: *Modern Pharmaceutics* Edited by G.S. Banker and C.T. Rhodes Chapter 10,

10   1979, herein incorporated by reference. In general, the formulation will include the

component or components (or chemically modified forms thereof) and inert ingredients

which allow for protection against the stomach environment, and release of the biologically

active material in the intestine.

In a further embodiment of the present invention, methods and agents are provided for the

15   augmentation of the migration of monocytes, by enhancing the functioning of the monocyte

membrane protein p-glycoprotein. The selection of suitable agents and routes of delivery are

as those described above in relation to modulating the same membrane protein on dendritic

cells. This aspect of the invention has utility in the treatment of various disorders, preferably

chronic inflammatory conditions which are not necessarily, but may be, initiated or

20   promulgated by antigens and antigen-specific immune cells such as cytotoxic T lymphocytes.

For example, the development and progression of atherosclerotic lesions involves the

progressive accumulation and retention of monocytes (which become tissue macrophages)

and other cell types in lesions; the local inflammation mediated by their secretory products

which attracts more pro-inflammatory cells, together with secretion of growth factors, is a pathophysiologic basis for vessel occlusion.

The methods and agents of the present invention directed to increasing monocyte migration has particular utility in preventing the retention of monocytes in inflammatory sites within the

5   body, thus limiting the extent of inflammation. Other examples of diseases and condition in which accumulation and retention of monocytes and resultant production of inflammatory mediators, growth factors and chemoattractants for additional inflammatory cells include rheumatoid arthritis and the granulomatous diseases.

It is a further object of the present invention to provide methods for identifying agents and

10   compound useful in modulating the migration of dendritic cells. As a consequence of the discovery by the inventors herein of the presence of p-glycoprotein on the membrane of dendritic cells and the further discovery of the role of p-glycoprotein and tissue factor in dendritic cell migration, various known methods for measuring the effect of compounds on p-glycoprotein activity may be adapted for use on dendritic cells. For example, dendritic cells

15   may be isolated from skin explants and loaded with a dye known to be exported by functioning p-glycoprotein (28), such as 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$). Export of the dye may be measured in the absence and presence of compounds suspected of inhibiting or enhancing the activity of p-glycoprotein by decreasing or increasing, respectively, the pumping of the dye out of the cell. In a further embodiment, agents useful

20   for the modulation of dendritic cell migration may be identified by first identifying whether a candidate agent is capable of interacting with p-glycoprotein or tissue factor; and subsequently determining the extent of modulation of the agent on the migration of dendritic

cells in vitro or in vivo. The initial screen may be performed on cells which express p-glycoprotein, such as any one of numerous cancer cell lines.

Subsequently, the effect of the compound on the migration of dendritic cells may be

5   performed as described below or that described in U.S. Patent 5,627,025, incorporated herein
         by reference. This method employs human skin explants in culture to measure the effect of
         compounds on dendritic cell emigrating from the skin explant into the culture medium. The
         methods disclosed herein are not limited to any particular method but are based upon the
         newly-identified role of p-glycoprotein and tissue factor on dendritic cells in cell migration.

Likewise, assays may be performed to identify compounds which enhance migration of

10   monocytes out of tissues. As one non-limiting example, migratory behavior of monocytes
         may be evaluated in an in vitro model of the blood vessel wall consisting of human umbilical
         vein endothelial cells (HUVEC) grown on type I collagen gels (see Example I, below).
         Human blood monocytes, which express p-glycoprotein, transmigrate across the confluent
         endothelium and enter the collagenous substrate. The extent of migration is measured after

15   exposure of the monocytes to the candidate. Other methods of measuring the migration of
         monocytes are known to the skilled artisan and are useful in the practice of the present
         invention.

In a further example of a method for identifying compounds useful for modulating dendritic
cell p-glycoprotein activity, the following steps are carried out:

20   i)   loading isolated dendritic cells with a detectable substrate of the p-
                                  glycoprotein transporter activity;

ii)   exposing the loaded cells to an agent suspected of modulating p-
        glycoprotein activity;

iii)   measuring the rate of transport of the detectable substrate from the
        dendritic cells; and

5   iv)   comparing said rate to the rate of p-glycoprotein transporter activity
        determined of control dendritic cells not exposed to the agent.

An increase or decreased in the transport rate in the presence of the agent indicates the extent

of activity of the agent in increasing or decreasing, respectively, p-glycoprotein activity of

said dendritic cells. In a further step, compounds that are active in the above assay, either at

10   increasing or decreasing p-glycoprotein activity, can be additionally assays to determine if

they are useful for modulating the migration of dendritic cells by determining the extent of

modulation of the agent of the migration of dendritic cells in vitro or in vivo. DC migration

may be determined by any of several techniques, such as the emigration rate described in

aforementioned U.S. Patent 5,627,025. In another method, compounds may be evaluated

15   directly in the emigration assay (7). Activity may be evaluated in vivo by administration of

the agent to an animal, followed by, for example, application of an antigen to the skin which

would under normal circumstances induce immunity in the animal or an immune response.

The extent of the any reduction in the immune response elicited after administration of the

agent is an indication of the extent of inhibition of dendritic cell migration. Similarly,

20   compounds to be evaluated for the enhancement of the immune response may be evaluated in

vivo by administering the agent parenterally or to the skin, for example, followed by

application of an immunogen. Any resulting enhancement of the immune response as

compared with control animals indicates positive activity in enhancing the immune response.

The following examples are presented in order to more fully illustrate the preferred

embodiments of the invention. They should in no way be construed, however, as limiting the

broad scope of the invention.

MATERIALS AND METHODS

5   *Transendothelial Migration Assays* Using a method detailed previously (10, 11), freshly

isolated peripheral blood mononuclear cells (PBMC), approximately one-fourth of which are

monocytes, were added to confluent monolayers of unstimulated HUVEC grown on type I

bovine collagen gels with or without addition of mAb. Cultures were incubated for 1.5 hr;

during this time, monocytes, but very few lymphocytes, transmigrate into the collagen (10).

10   To examine their subsequent basal-to-apical transendothelial migration, monocytes were

allowed to accumulate beneath the endothelium in the absence of added mAb. Then

monolayers were washed twice in Medium 199 (Life Technologies, Grand Island, NY) to

remove nonadherent cells from the apical surface, and individual wells received aliquots of

Medium 199 containing 20% heat-inactivated human serum with or without added

15   monoclonal antibodies (mAbs) as indicated. After 24 hr of incubation, cultures were washed

twice in Medium 199 and control medium or medium containing mAb was replenished.

After a total time of 48 hr in culture, samples were fixed for microscopic analysis.

Differential interference contrast optics were used to quantitate the number of mononuclear

phagocytes (MP) that were beneath the endothelial monolayer (11) in at least five high-power

20   fields per sample. Each experiment was conducted with six replicates per parameter tested.

Skin Cultures Human split-thickness skin was obtained from the New York Firefighter's Skin

Bank (New York Hospital-Cornell Medical Center) from cadavers within 24 hr of death, or

from patients undergoing plastic surgery, and was authorized for use in research. Generally,

dermatomes were approximately 300-mm thick, including both epidermis (about 100-mm)

and a portion of the dermis. Skin was prepared, cultured, and emigrated cells were

quantitated as previously described (7). Each explant was trimmed to 400-mm^2 and floated in

5   3 to 6 ml culture medium. When used, mAbs or verapamil (Sigma) were added to the culture

medium at the onset of culture, and cultures were incubated undisturbed until the indicated

day of analysis. Verapamil was prepared as a concentrated stock in methanol. Final

methanol concentration in cultures was 0.03% (vol/vol).

Immunostaining and Flow Cytometry Skin explants were separated into epidermal and

10   dermal sheets by treatment with 0.5 M ammonium thiocyanate for 20 minutes at 37° C (12).

Alternatively, skin samples were snap-frozen in OCT compound (Miles; Elkhart, IN) and

then used for the preparation of thick sections (approximately 100 mm) cut parallel to the

epidermis (6). After either procedure, sheets of skin were fixed in acetone for 5 min. before

staining. Sheets of epidermis or thick sections were incubated overnight at 4°C in Eppendorf

15   tubes with CY3-conjugated goat anti-mouse IgG (1:200; Jackson Immunoresearch

Laboratories, Inc.),1:20 FITC-conjugated anti-MHC II (Becton Dickinson), or anti-MHC II

9.3C9 hybridoma supernatant. Tissues were washed three times in PBS containing 1%

Tween 20, 15 min. each at room temperature. For detection of 9.3C9 when it was used,

CY3-conjugated goat anti-mouse IgG (Jackson Immunoresearch, Inc.) was added for 2 hr at

20   4° C, followed by three washes as just described. Staining for flow cytometry was carried out

as previously detailed (7).

In some experiments, explants were incubated in dispase II (Boehringer Mannheim) for 45

min. at 37°C to separate the epidermis from dermis. Pieces of epidermis were submerged in

0.05% trypsin/0.53 mM EDTA for 45 min. Single cell suspensions were generated by gently

pressing epidermal pieces through a fine mesh. Aliquots of digested cells were incubated

5   with FITC-labeled anti-MHC II for 30 min. on ice, washed twice, and analyzed by flow

cytometry.

Immunoblots Emigrated DC were purified by negative selection: anti-CD3 mAb was

incubated with emigrated cells, and T lymphocytes were removed using anti-mouse

IgG-conjugated magnetic beads (Dynal). The purity of the resulting population was verified

10   by flow cytometric evaluation after staining with PE-conjugated anti-MHC II (Becton

Dickinson). Microsomal membranes from 5 million thus purified DC, 25 million PBMC, or

5 million HUVEC were prepared as described (13), subjected to SDS-PAGE (4-12% gradient

gel) under reducing conditions, and electroblotted onto a nitrocellulose membrane. The

membrane was probed with 5 mg/ml C219 anti-MDR-1 mAb (Centocor) and visualized using

15   peroxidase-conjugated anti-mouse IgG, followed by ECL substrate (Amersham).

Efflux Assays A previously published method to detect functional MDR-1 (14) was

employed with minor modifications. Emigrated cells were incubated in RPMI-1640

containing 20 ng/ml $DiOC_2$ (Molecular Probes) for 15 min. at 37 °C, washed twice in ice cold

medium, resuspended in 10% fetal bovine serum/RPMI-1640 containing no additives or with

20   addition of 10 mg/ml UIC2 mAb (Coulter). After 90 min. of incubation at 37 °C, cells were

washed and immediately placed on ice for labeling with PE-conjugated anti-MHC II mAb

and analysis by flow cytometry.

EXAMPLE 1:   **Role of MDR-1 in basal-to-apical transendothelial migration of**

mononuclear phagocytes.

The present inventors were drawn to investigate the role of p-glycoprotein in dendritic cell

migration by a set of observations with blood monocytes, now known to be a progenitor of

5   DC when appropriately stimulated with cytokines (15-19). In these initial experiments, the

migratory behavior of monocytes was examined in an in vitro model of the blood vessel wall

consisting of human umbilical vein endothelial cells (HUVEC) grown on type I collagen gels

(10, 11). Human blood monocytes, which express MDR-1 (20, and data not shown),

transmigrate across the confluent endothelium and enter the collagenous substrate. This

10   initial migration into the collagen, which is completed within one hr, was unaffected by the

addition of anti-MDR-1 mAb, but was partially blocked by mAbs against the β_2 chain of

integrins (CD18) and CD31, as described (10, 11) (**Fig. 1A**).

After a brief residence in the collagen, a majority of subendothelial MP retraverse the

overlying, intact endothelium with a $t_{1/2}$ of 24 to 48 hr (21). This reverse transendothelial

15   migration was not prevented by mAb to CD18 or CD31 (**Fig. 1B**). However, screening of

mAbs (> 100) submitted to the VIth International Workshop for Human Leukocyte

Differentiation Antigens revealed that neutralizing mAbs to MDR-1 strongly inhibited

reverse transmigration. UIC2 (22), 4E3 (23), and MRK16 (24) against extracellular domains

of MDR-1 inhibited reverse transmigration by 50%, 64%, and 88%, respectively (**Fig. 1B**).

20   MRK16, used here as $F(ab')_2$ fragments, was the most potent blocking mAb, exhibiting

maximal levels of inhibition using as little as 0.5 to 1.0 mg/ml. C219 (25), which binds an

intracellular epitope of MDR-1, had no effect. Preincubation of monocytes with MRK16,

and subsequent removal of unbound mAb before addition of these cells to the endothelium

blocked reverse transmigration as well as when the mAb was present continuously during the

assay (Pre-MRK16, **Fig. 1B**). No other mAbs screened inhibited reverse transmigration,

except one against tissue factor.

5   **EXAMPLE 2:   Role of MDR-1 in mobilization of DC and T lymphocytes from**

skin.

During reverse transmigration, MP migrate across endothelium in a basal-to-apical direction,

a movement that is reminiscent of the normal trafficking of DC from the periphery into

afferent lymphatics. A panel of mAbs, including those against MDR-1, was applied to

10   cultured human skin explants. Appearance of DC and T lymphocytes in the culture medium

of the explants was reduced by 71 ± 9%, and 79 ± 16% when the skin was incubated in the

presence of MRK16 at concentrations of 2 and 10 mg/ml, respectively **(Fig. 2A)**. Verapamil,

a drug that antagonizes MDR-1 transport (26), reduced DC and T lymphocyte accumulation

in the culture medium by 52 ± 19% at 10 mg/ml (20 mM) **(Fig. 2A)**. The anti-MDR-1 mAb

15   UIC2 also blocked migration, by 75 ± 2% when used at 2 mg/ml **(Fig. 2A)**. An isotype

matched control mAb against cadherin 5 (Hec 1) (11) that does not react with DC or T cells

had no effect. Moreover, isotype-matched mAbs against other DC surface proteins--CD18

(IB4), CD31 (hec7), and MHC I (W6/32)--also did not inhibit the migration significantly

(Fig. 2B). Under all conditions, the cellular content of émigrés ranged from 40 to 60% DC,

20   with the remaining population being T lymphocytes. Thus, antagonists of MDR-1 blocked

migration of DC and T lymphocyte uniformly. Cell viability in all groups was >90%, as

assessed by trypan blue exclusion.

EXAMPLE 3:   **Expression of MDR-1 by DC.**

T lymphocytes are known to express MDR-1 (14, 20, 27), but its expression by DC has not

been reported previously. Immunostaining for MDR-1 was observed on DC in the epidermis

(**Fig. 3A and C**). The same cells also stained positively for the DC marker MHC II (**Fig. 3B**

5   **and C**). No other cell types in the skin, such as keratinocytes or fibroblasts, showed positive

staining for MDR-1 in these experiments. For these studies, skin was cultured in the

presence of MRK16 for two days, as for migration analysis. Then staining was carried out on

tangential sections prepared from snap-frozen skin, applying only anti-mouse Cy3-conjugated

detection antibody. This approach illustrates not only the presence of MDR-1 on epidermal

10   DC in situ, but also demonstrates that mAb applied during culture gains sufficient access to

the epidermis. Skin incubated with isotype-matched control mAb hec 1 did not show positive

staining.

Flow cytometric analysis of the cells that emigrated from control explants using double

labeling with anti-MDR-1 (using either MRK16 or UIC2) and anti-MHC II mAbs

15   demonstrated high levels of MDR-1 on all DC in some experiments (**Fig. 4A**) or on a

majority, but not all, DC in other experiments (**Fig. 4B**). The fraction of DC staining

positively was always greater than the fraction of positively stained subset of T lymphocytes

(**Fig. 4C**).

Expression of authentic MDR-1 by DC was evident in two assays. To document expression

20   of functional MDR-1 (28), DC were loaded with a synthetic, fluorescent substrate for

MDR-1, $DiOC_2$. At 37 °C, but not 4 °C, DC transported this substrate into the surrounding

medium, showing a log decrease in fluorescence intensity (**Fig. 4D**), and this efflux was

-34-

inhibited by anti-MDR-1 mAb UIC2 **(Fig. 4D)**. To identify MDR-1 molecules in DC, crude

membranes were prepared and immunoblotted using mAb C219, another mAb that reacts

with MDR-1. A broad band of approximately 170 to 180 kDa, the reported molecular weight

of MDR-1 was identified **(Fig. 4E)**. The band observed matched that seen in membranes

5   from PBMC and was not observed in HUVEC **(Fig. 4E)**, which do not express MDR-1.

EXAMPLE 4:   **Effect of Verapamil on the DiOC$_2$ Efflux from Dendritic Cells**

Figure 5 further documents that functional p-glycoprotein is expressed by dendritic cells and

that p-glycoprotein-mediated transport can be blocked by agents, particularly verapamil and a

monoclonal antibody against p-glycoprotein, that are known to inhibit p-glycoprotein

10   transport activity. As described in the Brief Description of **Figure 5**, dendritic cells were

loaded with the p-glycoprotein synthetic substrate 3,3'-diethyloxacarbocyanine iodide (DiOC$_2$

), and flow cytometry performed as described in Example 3. Approximately 90% of the dye

was transported out of the dendritic cells incubated in the absence of p-glycoprotein

inhibitors, as indicated by the one-log decrease in fluorescence intensity in the FL1 channel

15   (x-axis) (compare Panel C to D). However, transport of the dye was completely prevented in

the presence of verapamil (Panel E) and the anti-p-glycoprotein monoclonal antibody UIC2

(Panel F).

Example 5:   **Effect of MDR-1 antagonists on maturation and retention of DC in**

                  epidermis.

20   To examine the step at which emigration of DC from the explants was inhibited by

anti-MDR-1 mAb, epidermal and dermal sheets prepared from cultured skin explants were

stained for MHC II (29, 30). Compared to the number present at the onset of culture **(Fig.**

6A, B), the number of DC remaining in the epidermis after three days of culture under control

conditions was decreased by 56% **(Fig. 6A, C)**. In explants cultured in the presence of

MRK16 **(Fig. 6A, D)**, UIC2 **(Fig. 6A)**, or verapamil **(Fig. 6A)**, a more limited reduction in

DC density of 13%, 11%, and 25%, respectively, were observed. Thus, antagonism of

5   MDR-1 results in retention of DC in the epidermis.

In cultures treated with anti-MDR-1 mAb MRK16, some DC retained in the epidermis

appeared morphologically similar to epidermal DC before the onset of culture (compare **Fig.**

6B and D), but others showed evidence of maturation resembling that observed under control

conditions **(Fig. 6C** and data not shown), including an increase in cell size and greater

10   intensity of staining for MHC II (4, 29, 30). Flow cytometric analysis of DC enzymatically

removed from the epidermis after culture in the presence of anti-MDR-1 mAb MRK16 or

under control conditions indicated that the maturation of DC, which usually accompanies

their mobilization, was not inhibited by anti-MDR-1, as exemplified by the up regulation of

MHC II in the majority of DC in the presence or absence of MRK16 **(Fig. 7)**. Thus,

15   antagonism of MDR-1 strongly impedes the migration of DC, but only inconsistently or

negligibly suppresses their maturation.

EXAMPLE 6:   **Effect of Anti-Tissue Factor Antibodies on Emigration of**

                       Dendritic Cells and T Lymphocytes from Skin Explants

Explants of human skin were floated in culture medium without added monoclonal antibody

20   or in medium containing anti-tissue factor monoclonal antibody VIC7 or control antibodies

against cadherin 5 or carcinoembryonic antigen (CEA). After three days of incubation, DC

and T lymphocytes that appeared in the culture medium were collected. Each experiment

was conducted with 3-4 replicates per parameter tested. The number of emigrated cells

recovered from individual control explants was typically 5 x 10⁵. To compare data from

different experiments, the mean number of emigrated cells in the absence of added

5   monoclonal antibody in each experiment was set equal to 1.0 and relative values were

obtained for the remaining data. Cumulative results from three independent experiments are

shown in **Figure 8**. These experiments show that monoclonal antibody to tissue factor

reduces the emigration of cells from skin explants by about 50%.

EXAMPLE 7:   Effect of P-glycoprotein Antagonists on Dendritic Cell Migration

10   **in Vivo**

To test the effectiveness of p-glycoprotein antagonists on dendritic cell migration in vivo,

experiments were carried out in mice. First, the murine molecule analogous to p-

glycoprotein in mouse DCs was identified. To do this, mouse skin explants, derived from

the dorsal aspect of the ear, were cultured in a manner similar to the human skin explants

15   (50). Dendritic cells (DCs) that migrated out of the lymphatic vessels of these explants

were used in efflux transport assays and in flow cytometry. Efflux of the fluorescent

substrate Fluo-3 for p-glycoprotein-related molecules was observed (**Fig. 9A**, left panel),

and flow cytometric evaluation using specific antibodies demonstrated the expression of

murine multidrug resistance related protein (MRP) (**Fig. 9B**). That MRP was the major p-

20   glycoprotein-related transporter was further evident from the observation that MRP knock-

out mice (51) failed to efflux the fluorescent substrate Fluo-3 (**Fig. 9A**, right panel).

The pharmacologic compound MK 571 is a substrate that antagonizes p-glycoprotein and

MRP, but is a particularly potent inhibitor of the latter (52). Thus, MK 571 was added to

cultures of human skin explants and mouse ear explants. Migration of DC was blocked by

58% and 62%, respectively (**Figs. 10 A,B**).

5   MRP knock-out mice and age-matched wild-type mice were employed in a contact

sensitivity assay to determine whether MRP mediated the migration of murine DCs in vivo.

Dendritic cells were analyzed for their transport of the contact sensitizer FITC to lymph

nodes 24 h after its application to the skin of mice, using the contact sensitivity assay

previously described (53). Dendritic cell accumulation in the lymph nodes of sensitized

10   mice was reduced by 87% in the absence of MRP expression (**Fig. 11A**).

Next, using wild-type mice, MK 571 was administered i.v. (50 mg / kg body weight) four

hours prior to application of the contact sensitizer FITC to determine whether

pharmacologic inhibitors of MRP and p-glycoprotein are effective in suppressing DC

migration. Indeed, administration of MK 571 reduced accumulation of FITC-bearing DCs

15   in the draining lymph node by 64% (**Fig. 11B**). These data indicate that pharmacologic

antagonism of p-glycoprotein suppresses dendritic cell migration.

This invention may be embodied in other forms or carried out in other ways without

departing from the spirit or essential characteristics thereof. The present disclosure is

therefore to be considered as in all respects illustrative and not restrictive, the scope of the

invention being indicated by the appended Claims, and all changes which come within the

meaning and range of equivalency are intended to be embraced therein.

Various publications are cited herein, the disclosures of which are incorporated by reference

in their entireties.

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WHAT IS CLAIMED IS:

1.   1.   A method for decreasing the migration of dendritic cells from the peripheral tissues to

2   the lymphatic vessels by contacting said dendritic cells with an effective amount of an

3   agent capable of inhibiting the activity of a dendritic cell membrane protein selected

4   from the group consisting of p-glycoprotein, tissue factor and the combination

5   thereof.

1.   2.   The method of claim 2 wherein said agent is an antibody which binds to p-

2   glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which

3   blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an

4   antisense oligonucleotide to p-glycoprotein.

1.   3.   The method of claim 3 wherein said antibodies or antibody fragments which bind to

2   p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and

3   antibody fragments thereof which bind p-glycoprotein.

1.   4.   The method of claim 1 wherein said agent is capable of reversing multidrug

2   resistance.

1.   5.   The method of claim 1 wherein said agent is a calcium channel blocker, reserpine,

2   quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,

3   yohimbine, phenothiazines, tamoxifen, N-desmethylramoxifen, 4-hydroxytamoxifen,

4   toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5   gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,

6   cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,

7   rapamycin, MK 571, or SR33557.

1   6.   The method of claim 5 wherein said calcium channel blocker is selected from the

2   group consisting of verapamil, diltiazem, azidopine, dihydroperidine, dexverapamil,

3   nifedipine, bepridil, nicardipine, dexniguldipine, and combinations thereof.

1   7.   The method of claim 5 wherein said cyclosporin is cyclosporine A, staurosporine,

2   PSC 833, and combinations thereof.

1   8.   The method of claim 5 wherein said phenothiazine is selected from the group

2   consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1   9.   The method of claim 1 wherein said agent is an antagonist of tissue factor.

1   10.   The method of claim 9 wherein said agent is an antibody or an antibody fragment to

2   tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a

3   recombinant fragment of tissue factor.

1   11.   The method of claim 9 wherein said agent is selected from the group consisting of

2   dilazep and retinoic acid.

1   12.   A method for suppressing the development of immunity or an immune response in a

2   mammal by contacting dendritic cells of said mammal with a therapeutically effective

3   amount of an agent capable of inhibiting the activity of a dendritic cell membrane

4   protein selected from the group consisting of p-glycoprotein, tissue factor and the

5   combination thereof.

1   13.   The method of claim 12 wherein said agent is an antibody which binds to p-

2   glycoprotein, an antibody fragment which binds to p-glycoprotein, an agent which

3   blocks transport activity of p-glycoprotein, an antagonist of p-glycoprotein, or an

4   antisense oligonucleotide to p-glycoprotein.

1   14.   The method of claim 13 wherein said antibodies or antibody fragments which bind to

2   p-glycoprotein are selected from the group consisting of MRK-16, UIC2, 4E3, and

3   antibody fragments thereof which bind p-glycoprotein.

1   15.   The method of claim 12 wherein said agent is capable of reversing multidrug

2   resistance.

1   16.   The method of claim 12 wherein said agent is a calcium channel blocker, reserpine,

2   quinine, quinidine, quinacrine, cinchonine, cyclosporine and cyclosporine analogs,

3   yohimbine, phenothiazines, tamoxifen, N-desmethylramoxifen, 4-hydroxytamoxifen,

4   toremifene, progesterone, cephalosporin antibiotics, erythromycin, valinomycin,

5   gramicidin, bafilomycin, amiodarone, dipyridamole, quinidine, cortisol, terfenadine,

6   cremophor, benzquinamide, estramustine, monensin, tacrolimus, GF120918, S9788,

7   rapamycin, MK 571, or SR33557.

1   17.   The method of claim 16 wherein said calcium channel blocker is selected from the

2   group consisting of verapamil, diltiazem, azidopine, dihydroperidine, dexverapamil,

3   nifedipine, bepridil, nicardipine, dexniguldipine, and combinations thereof.

1   18.   The method of claim 16 wherein said cyclosporin is cyclosporine A, staurosporine,

2   PSC 833, and combinations thereof.

1   19.   The method of claim 16 wherein said phenothiazine is selected from the group

2   consisting of trifluoperazine, chlorpromazine, fluphenazine, and combinations thereof.

1   20.   The method of claim 12 wherein said agent is an antagonist of tissue factor.

1   21.   The method of claim 20 wherein said agent is an antibody or an antibody fragment to

2   tissue factor, a tissue factor antisense oligonucleotide, a fragment of tissue factor, or a

3   recombinant fragment of tissue factor.

1   22.   The method of claim 20 wherein said agent is selected from the group consisting of

2   dilazep and retinoic acid.

1   23.   The method of claim 12 wherein said suppression of immunity or the immune

2   response inhibits the immune response to an allergen.

1   24.   The method of claim 12 wherein said suppression of immunity or the immune

2   response is used for the treatment of organ transplant rejection, graft-vs.-host disease,

3   autoimmune diseases, atopic diseases, or HIV infection.

1   25.   The method of claim 24 wherein said autoimmune diseases is rheumatoid arthritis or

2   psoriasis.

1   26.   The method of claim 24 wherein said atopic disease is contact dermatitis, food

2   allergy, allergic rhinitis, allergic conjunctivitis, asthma, or eczema.

1   27.   The method of claim 12 wherein said agent is administered by the topical, parenteral,

2   ophthalmic, nasal, rectal, bronchiolar or pulmonary route.

1   28.   The method of claim 27 wherein said parenteral administration intravenous,

2   intraarterial, intramuscular, subcutaneous, or intradermal.

1   29.   A method for increasing the migration of dendritic cells from the peripheral tissues to

2   the lymphatic vessels by contacting said dendritic cells with an effective amount of an

3   agent capable of increasing the activity of a dendritic cell membrane protein selected

4   from the group consisting of p-glycoprotein, tissue factor, and the combination

5   thereof.

1   30.   The method of claim 29 wherein said agent is a p-glycoprotein agonist or a tissue

2   factor agonist.

1   31.   The method of claim 30 wherein said agent is bromocriptine, N-acetyl-leucine$_n$-

2   tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl-γ,γ'-

3   dipyridylium dichloride.

1   32.   The method of claim 29 wherein said agent is co-administered with an immunogen to

2   enhance the development of immunity to said immunogen.

1   33.   The method of claim 32 wherein said immunogen is a vaccine.

1   34.   A method for enhancing the development of immunity or an immune response in a

2   mammal by contacting dendritic cells of said mammal with a therapeutically effective

3   amount of an agent capable of increasing the activity of a dendritic cell membrane

4   protein selected from the group consisting of p-glycoprotein, tissue factor, and the

5   combination thereof.

1   35.   The method of claim 34 wherein said agent is a p-glycoprotein agonist or a tissue

2   factor agonist.

1   36.   The method of claim 34 wherein said agent is bromocriptine, N-acetyl-leucine$_n$-

2   tyrosine amide where n=1-6, cytarabine, endotoxin, silver ion, or N,N'-dimethyl-γ,γ'-

3   dipyridylium dichloride.

1   37.   The method of claim 34 wherein said agent is co-administered with an immunogen to

2   enhance the development of immunity to said immunogen.

1   38.   The method of claim 37 wherein said immunogen is a vaccine.

2   39.   The method of claim 38 wherein said vaccine comprises a viral, bacterial, or parasite

3   immunogen.

1   40.   The method of claim 37 wherein said immunogen is a tumor antigen.

1   41.   A method for the identification of agents useful for the modulation of dendritic cell

2   migration comprising the steps of:

3   i)   identifying an agent capable of interacting with the a dendritic cell

4   membrane protein selected from the group consisting of p-glycoprotein

5   and tissue factor; and

6   ii)   determining the extent of modulation of said agent of step (i) in the

7   migration of dendritic cells in vitro or in vivo.

1   42.   A method for the identification of agents useful for modulating the development of

2   immunity or an immune response in accordance with the method of claim 41.

1   43.   A method for identifying compounds useful for modulating dendritic cell p-

2   glycoprotein activity comprising the steps of:

3   i)   loading isolated dendritic cells with a detectable substrate of the p-

4   glycoprotein transporter activity;

5   ii)   exposing said loaded cells to an agent suspected of modulating p-

6   glycoprotein activity;

7   iii)   measuring the rate of transport of said detectable substrate from said

8   dendritic cells; and

9   iv)   comparing said rate to the rate of p-glycoprotein transporter activity

10   determined of control dendritic cells not exposed to said agent,

11   wherein an increase or decreased in said rate in the presence of the agent indicates the

12   extent of activity of said agent in increasing or decreasing, respectively, p-

13   glycoprotein activity of said dendritic cells.

1   44.   A method for identifying compounds useful for modulating the migration of dendritic

2   cells by carrying out the method of claim 43, followed by determining the extent of

3   modulation of said agent of the migration of dendritic cells in vitro or in vivo.

1   45.   A method for augmenting the migration of monocytes and monocyte-derived cells

2   from tissues by contacting said monocytes with an effective amount of an agent

3   capable of augmenting monocyte p-glycoprotein activity.

1   46.   A method of treating a chronic inflammatory disease or condition in a mammal by

2   enhancing the migration of monocytes and monocyte-derived cells from the affected

3   site of said disease or condition by administration of a therapeutically effective

4   amount of an agent capable of augmenting monocyte p-glycoprotein activity.

1   47.   The method of claim 46 wherein said inflammatory disease or condition is

2   atherosclerosis, rheumatoid arthritis, or granulomatous diseases.

FIG. 1A

FIG. 1B
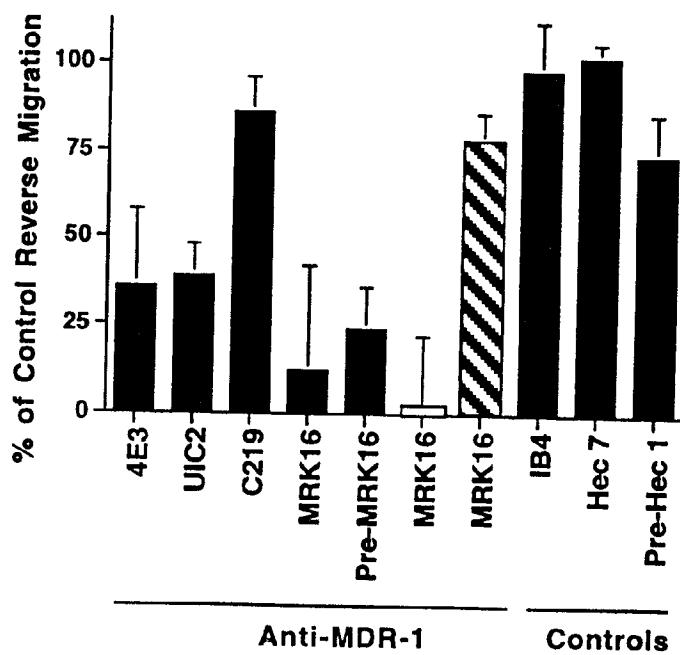
FIG. 2
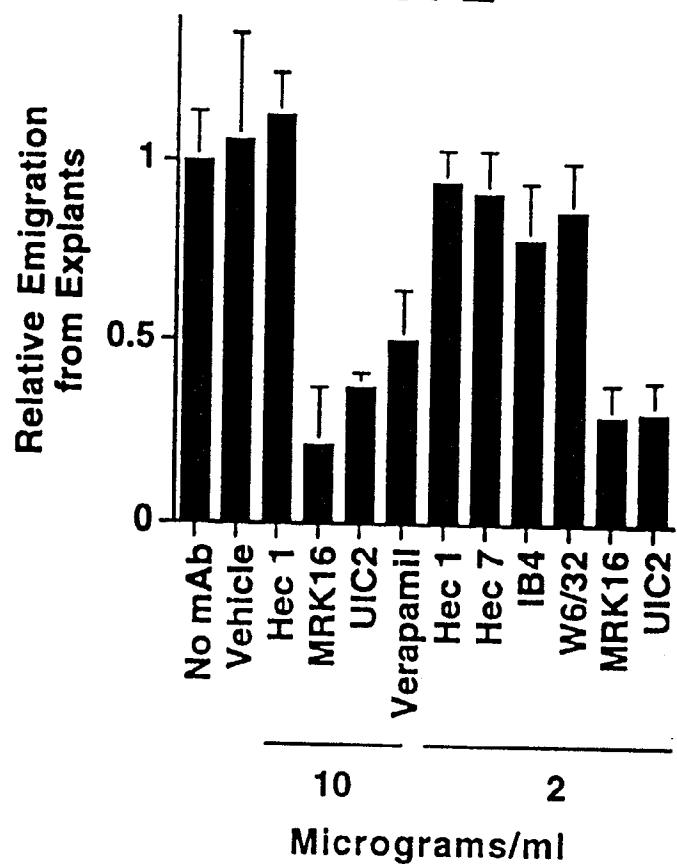
FIG. 3C

FIG. 3B

FIG. 3A

FIG. 4E

FIG. 4C

FIG. 4D

FIG. 4A

FIG. 4B

513
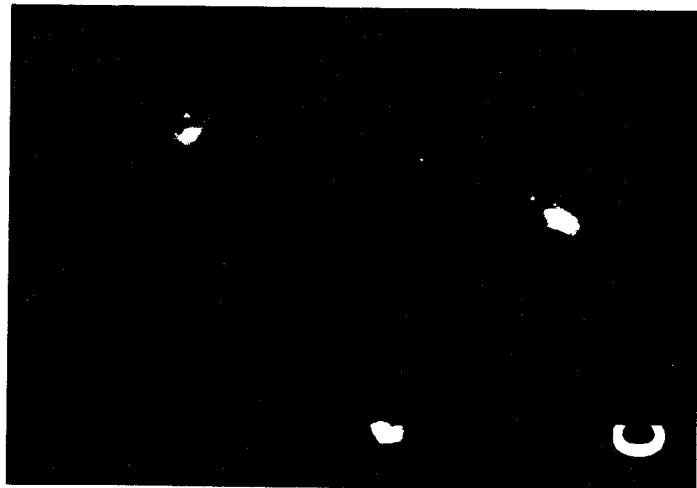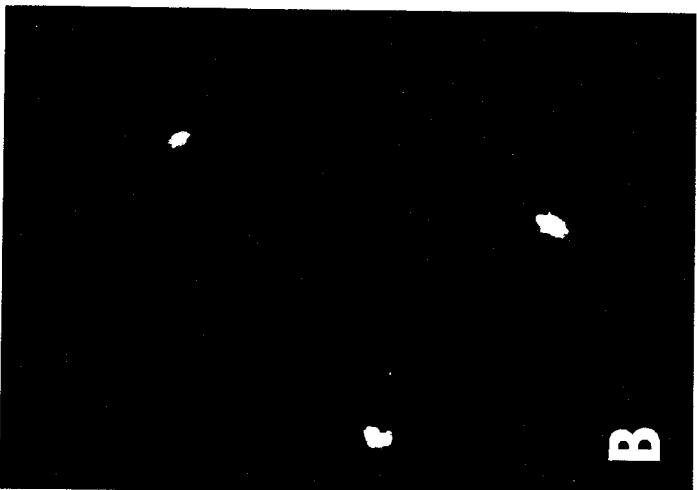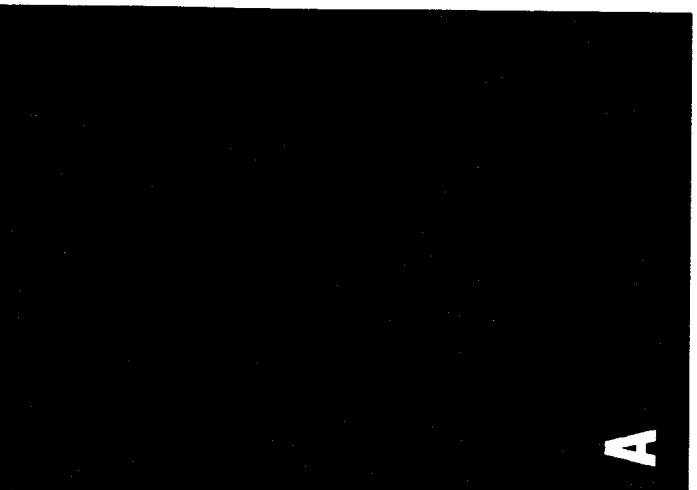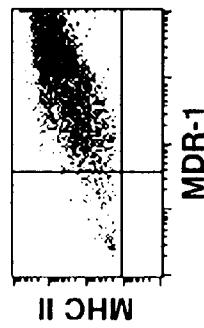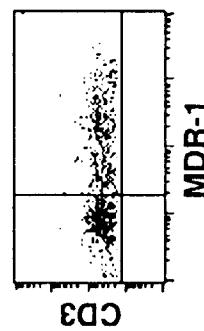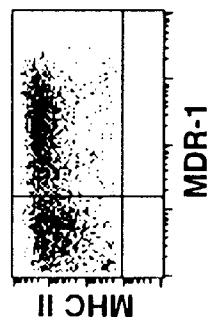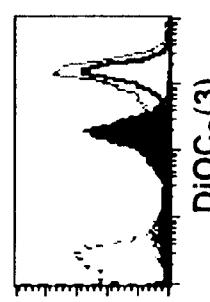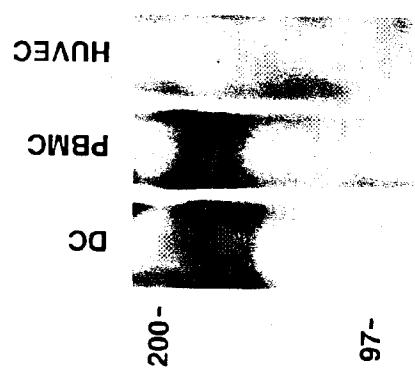
FIG. 5A
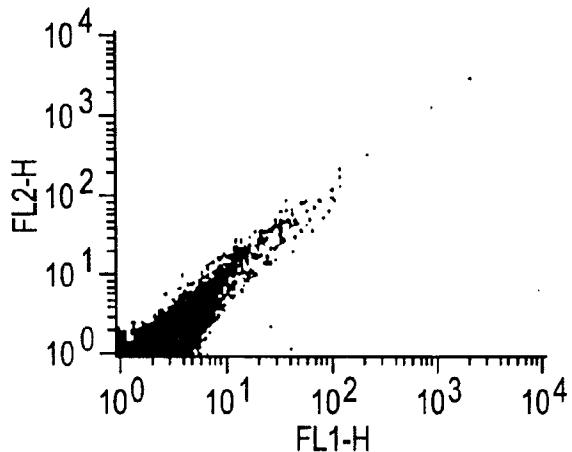
FIG. 5B
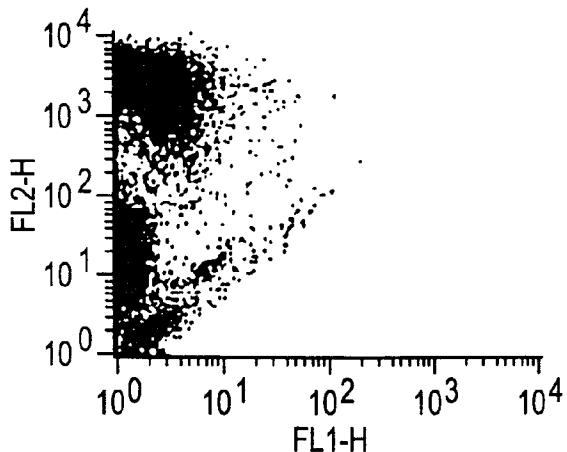
FIG. 5C
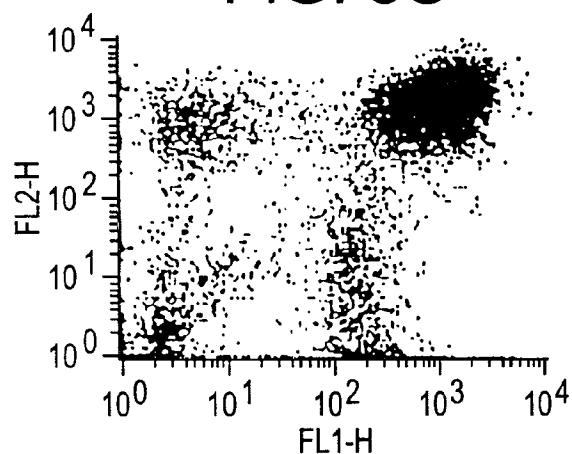
FIG. 5D
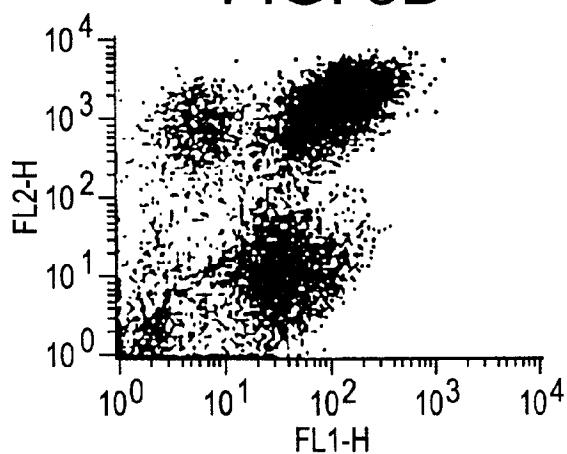
FIG. 5E
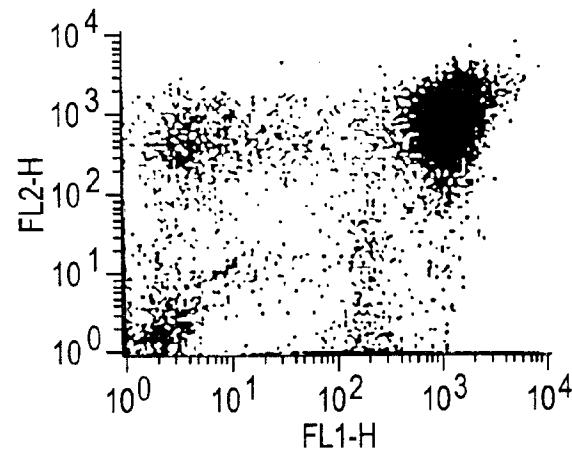
FIG. 5F
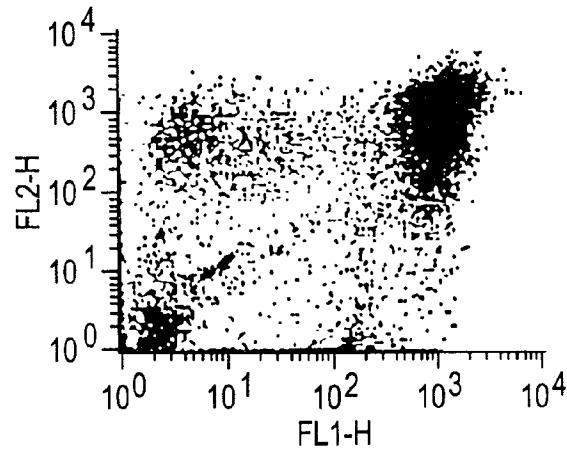
FIG. 6A
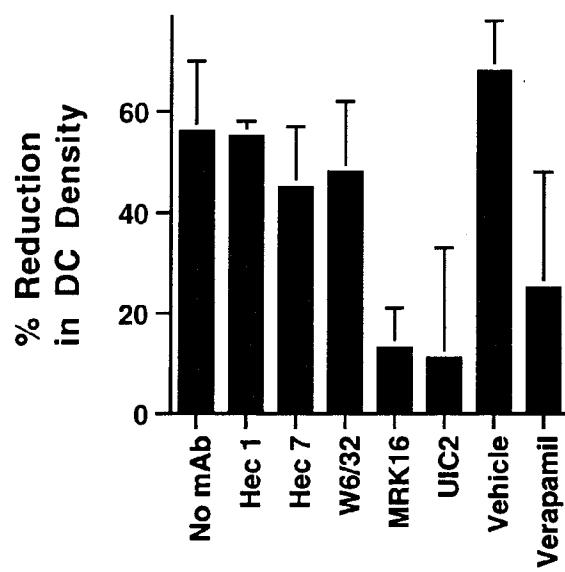
FIG. 6B

FIG. 6C

FIG. 6D

FIG. 7A

FIG. 7B

— Day 0   (Gated)
— Day 3, MRK16   (Gated)
█ Day 3, No mAb   (Gated)
---- Non-gated Keratinocytes

FIG. 8

FIG. 9A

Wild-type

Knock-out

FIG. 9B

— Negative Control
— Anti-MRP Antibody
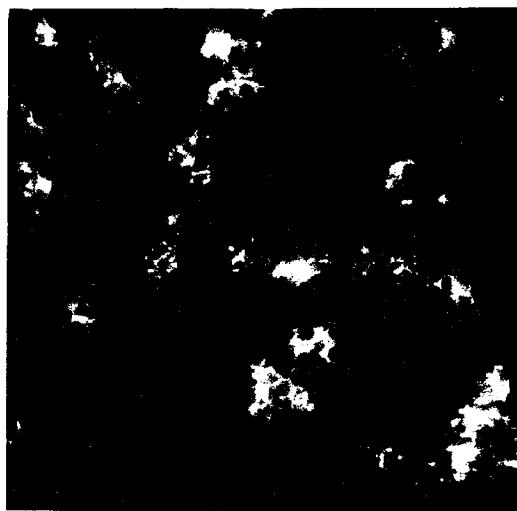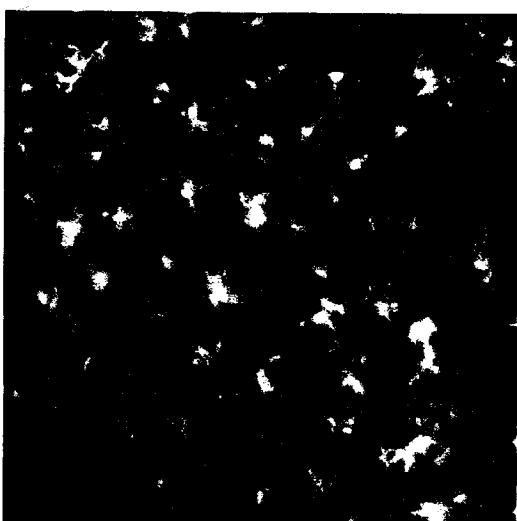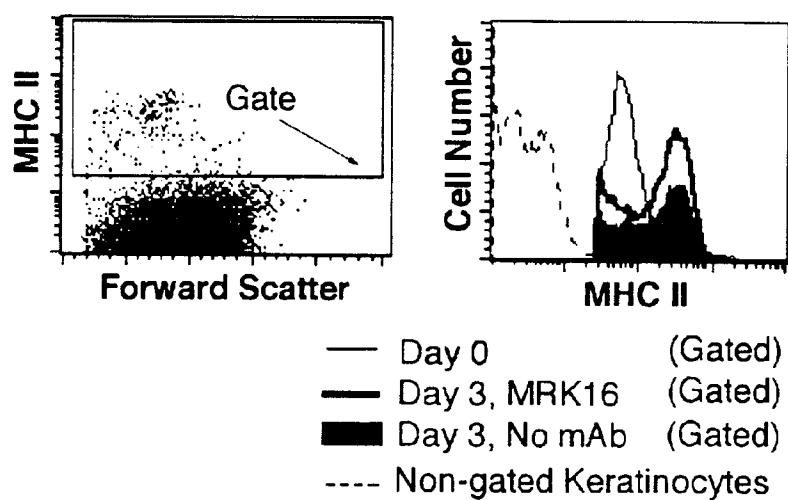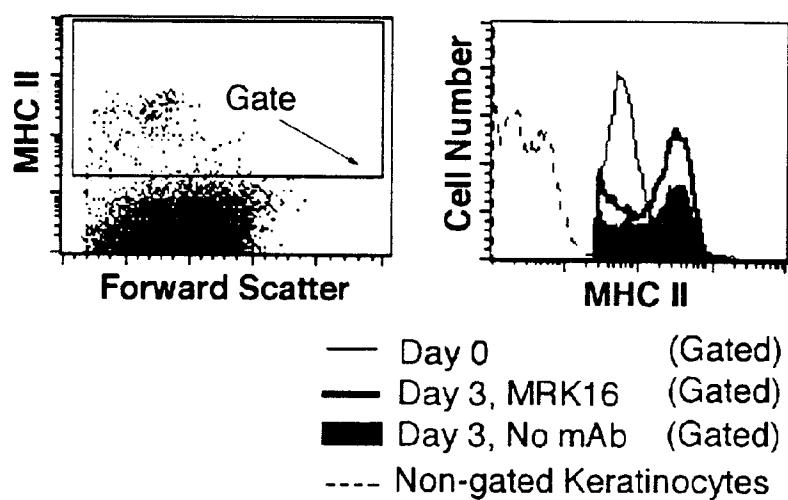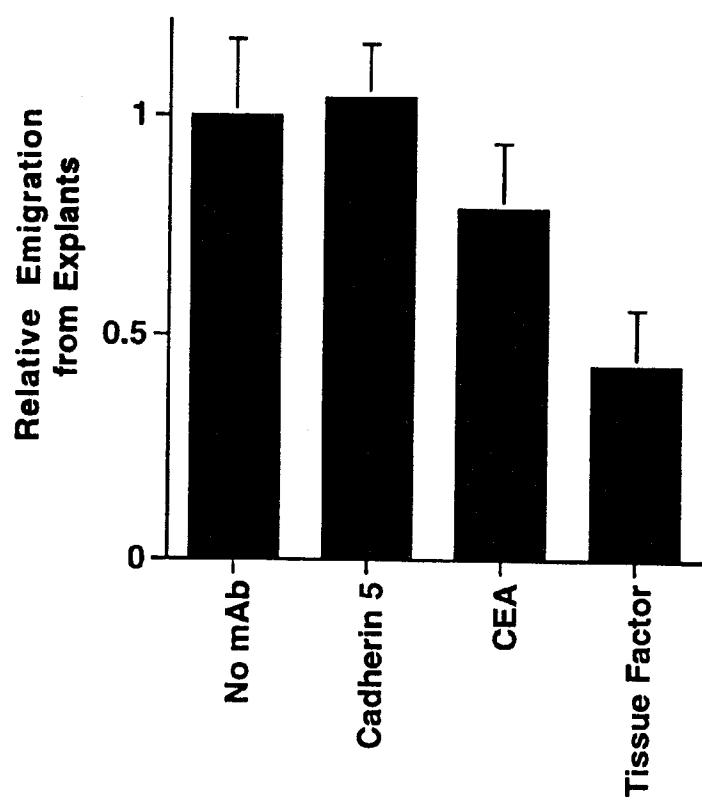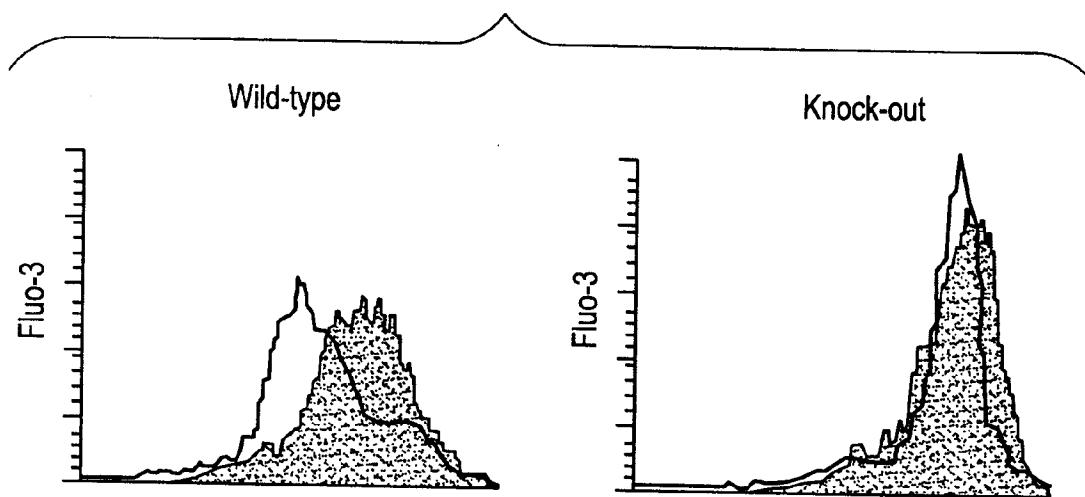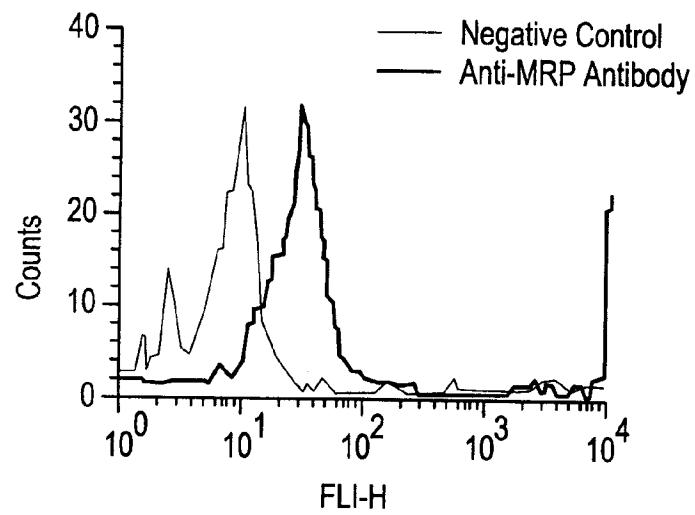
FIG. 10A

HUMAN
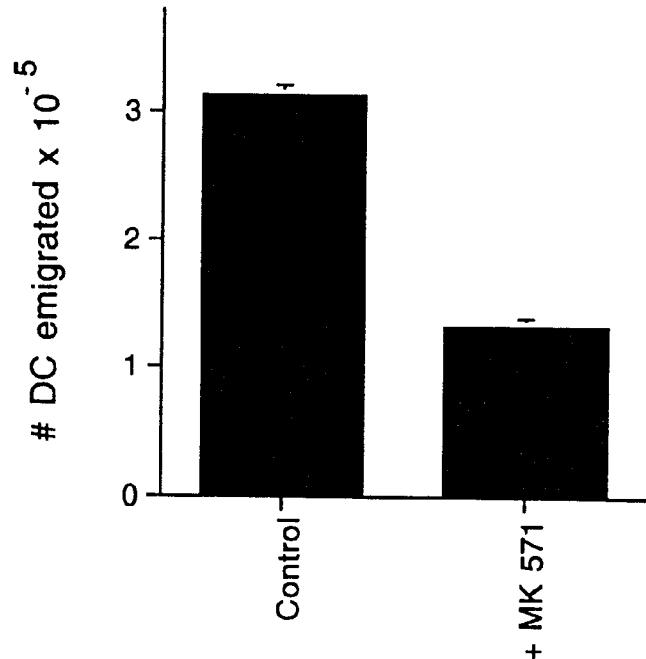
FIG. 10B

MOUSE
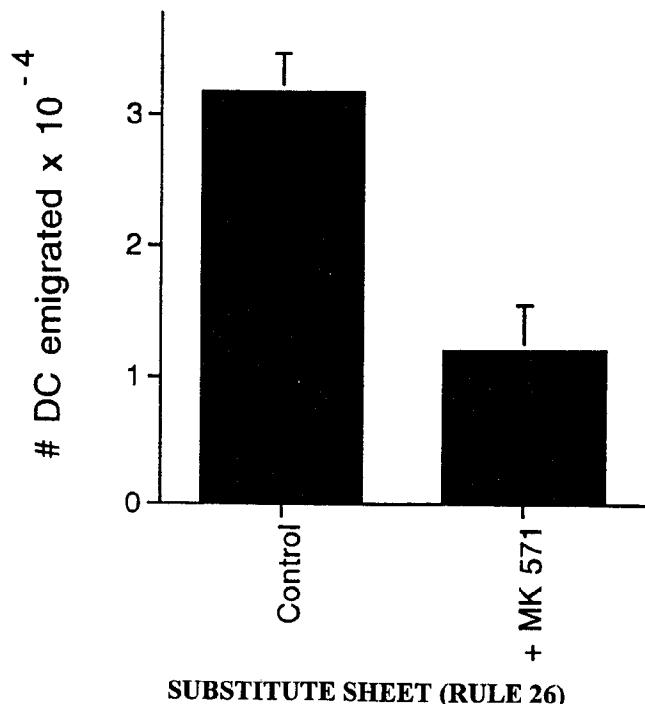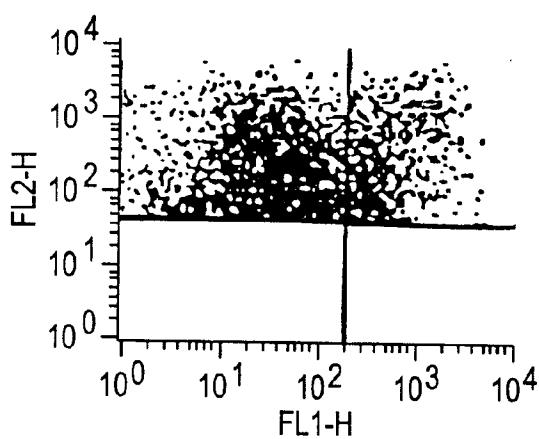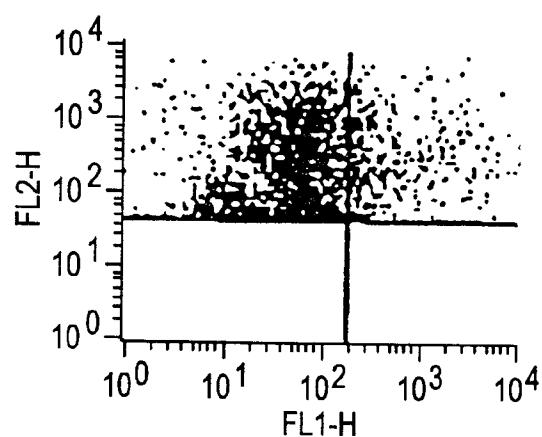
FIG. 11A
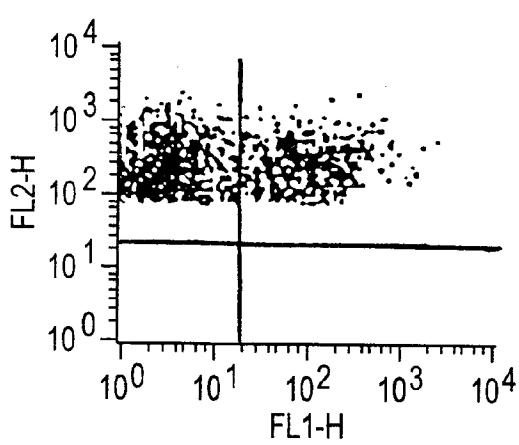
FIG. 11B

FIG. 11D

FIG. 11E
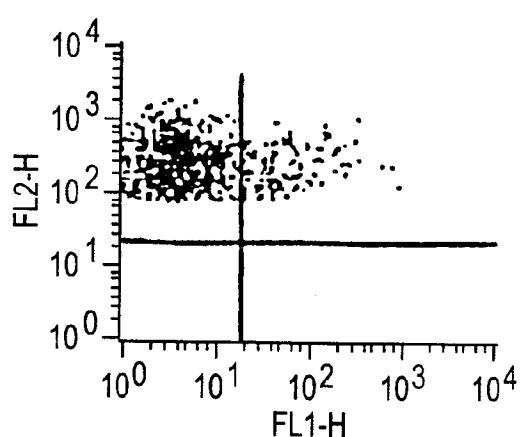
FIG. 11C

FIG. 11F

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K38/00   A61K39/395   C12N15/11   G01N33/53   A61K39/39
        A61K38/13   A61K38/36   A61K31/20   A61K31/275   C07D279/00
        A61K31/55   A61K39/00   A61K39/002   A61K39/12   A61K39/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RANDOLPH G J ET AL: "Role of tissue factor in adhesion of mononuclear phagocytes to and trafficking through endothelium in vitro." BLOOD, (1998 DEC 1) 92 (11) 4167-77. , XP002112753 abstract page 4167, left-hand column, paragraph 3 - right-hand column, paragraph 1 page 4169, right-hand column, paragraph 2 - page 4170, right-hand column, paragraph 3 page 4172, right-hand column, paragraph 2 - left-hand column, paragraph 4 page 4175, left-hand column, paragraph 1 - right-hand column, paragraph 1 page 4176, left-hand column, paragraph 1 --- -/--	1,9,10, 12,20, 21,23

| X | Further documents are listed in the continuation of box C. |
| X | Patent family members are listed in annex. |

° Special categories of cited documents :

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citation or other special reason (as specified)

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other means

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later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

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cannot be considered novel or cannot be considered to
involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1999	13/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   //C07K14/705,C07K14/745,C07K16/36,C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 12633 A (IMMUNEX CORP) 10 April 1997 (1997-04-10) page 2, line 31 - page 3, line 8 page 9, line 29-34 claims 16-19	29-40, 44,45
A	CUMBERBATCH M ET AL: "Tumour necrosis factor-alpha is required for accumulation of dendritic cells in draining lymph nodes and for optimal contact sensitization." IMMUNOLOGY, (1995 JAN) 84 (1) 31-5. , XP002112754 page 31, left-hand column, paragraph 1 - page 32, left-hand column, paragraph 1 page 32, left-hand column, paragraph 5 page 34, left-hand column, paragraph 1 - right-hand column, paragraph 2	1-27, 41-44, 46,47

-/--

| X | Further documents are listed in the continuation of box C. | X | Patent family members are listed in annex. |

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 August 1999	

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Covone, M

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2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 01645 A (PEPTECH UK LTD ;LEDGER PHILIP (GB)) 25 January 1996 (1996-01-25) page 1, line 6-22 page 22, line 23 - page 23, line 22 example 4 ---	1-27, 41-44, 46,47
A	MA J ET AL: "In vivo treatment with anti-ICAM-1 and anti-LFA-1 antibodies inhibits contact sensitization-induced migration of epidermal Langerhans cells to regional lymph nodes." CELLULAR IMMUNOLOGY, (1994 OCT 15) 158 (2) 389-99. , XP002112755 abstract page 392, paragraph 6 - page 394, paragraph 1 page 398, paragraph 3 ---	1-47
A	DATABASE MEDLINE 'Online! US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US LARSEN C P ET AL: "Migration and maturation of Langerhans cells in skin transplants and explants." retrieved from STN Database accession no. 91037811 XP002112758 abstract & JOURNAL OF EXPERIMENTAL MEDICINE, (1990 NOV 1) 172 (5) 1483-93. , ---	1-47
A	LAVIE Y. ET AL.: "Agents that reverse multidrug resistance, Tamoxifen, Verapamil, and Cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells" J. BIOL.CHEM., vol. 272, no. 3, 17 January 1997 (1997-01-17), pages 1682-87, XP002112756 abstract page 1686, paragraph 1 page 1687, paragraphs 2,3 ---	1,4-8, 12,15-19
P,X	RANDOLPH G J ET AL: "A physiologic function for p - glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 JUN 9) 95 (12) 6924-9. , XP002112757 the whole document -----	1-47

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Box I	Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

   1-40 and 45-47 (completely), 41-44 (partially)

   see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II	Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

Continuation of Box I.1

Although claims 41-44 (partially) are directed to a diagnostic method practised on the human/animal body and claims 1-40, 45-47 (completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 1-40 45-47 (completely), 41-44 (partially)

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy
Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9712633	A	10-04-1997	AU	697539 B	08-10-1998
			AU	7392296 A	28-04-1997
			CA	2232865 A	10-04-1997
			CZ	9800977 A	17-02-1999
			EP	0871487 A	21-10-1998
			LV	12085 A	20-07-1998
			LV	12085 B	20-09-1998
			NO	981374 A	03-06-1998
			PL	325964 A	17-08-1998
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			BG	101126 A	30-09-1997
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			CZ	9700047 A	16-07-1997
			EP	0768888 A	23-04-1997
			HU	77290 A	30-03-1998
			JP	10505580 T	02-06-1998
			SK	2697 A	06-08-1997